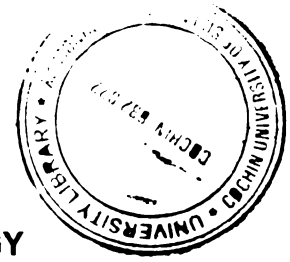
STUDIES ON ISOLATION, PURIFICATION AND PROPERTIES OF ENDOGLUCANASE FROM THE HEPATOPANCREAS OF *PERNA VIRIDIS*



THESIS SUBMITTED TO THE

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
COCHIN

IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

MARINE SCIENCES

BY
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COCHIN- 29

JULY 2004

CERTIFICATE

This is to certify that the thesis entitled "Studies on isolation, purification and properties of Endoglucanase from the hepatopancreas of *Perna viridis*" is an authentic record of the research work carried out by Smt. Sona. A, under my supervision and guidance in the Central Institute of Fisheries Technology, Cochin in partial fulfillment of the requirements for the degree of Doctor of Philosophy and that no part of this work thereof has been formed the basis of the award of any degree, diploma, associateship, fellowship or other similar titles of this or any other University or Society. She has also passed the Ph.D. qualifying examination of the Cochin University held in October 2003.

19-07-2004.

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DECLARATION

I, Sona.A, do hereby declare that the thesis entitled "Studies on isolation, purification and properties of Endoglucanase from the hepatopancreas of *Perna viridis*" is an authentic record of research work carried out by me under the supervision and guidance of Dr.M.K.Mukundan (Head, Quality Assurance and Management Division, Central Institute of Fisheries Technology) in partial fulfillment of the requirements for the Ph. D. Degree of Cochin University of Science & Technology and that no part thereof has been presented for the award of any other degree in any University.

Cochin-29
19-07-2004

(SONA. A.)

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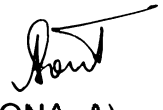

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ABBREVIATIONS

α	Alpha
β	Beta
µg	Microgram
µl	Microlitre
APS	Ammonium persulphate
A_{280}	Absorbance at 280nm
A°	Amstrong
BMCC	Bacterial microcrystalline cellulose
BSA	Bovine Serum Albumin
$CaCl_2.2H_2O$	Calcium chloride dihydtrate
CBH	Cellobiohydrolase
CMC	Carboxymethylcellulose
CO_2	Carbon dioxide
DEAE	Diethyl amino ethyl
DNSA	Dinitro salicylic acid
EDTA	Ethylene diamine tetra acetic acid
EG	Endoglucanase
g	Gram
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
IEC	Ion exchange chromatography
IEF	Isoelectric focusing
i.e.	that is
K_2SO_4	Potassium sulphate
K Da	Kilo Dalton
Km	Michaelis menten constant
mg	Milligram
ml	milli litre
min	Minutes
mM	Milli molar
MS	mass spectroscopy
N	Normal
NaOH	Sodium hydroxide
nm	Nanometer
No.	Number
OPA	o-phthalaldehyde
PAGE	Polyacrylamide gel electrophoresis
pI	isoelectric point
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SH	sulphydryl
[S]	Substrate concentration
TEMED	N, N, N′, N′ Tetramethyl ethylene diamine
Tris	Tris-(hydroxymethyl)amino methane
U	Units of endoglucanase Activity
UV	Ultra violet
Viz.	Namely
V	Velocity of reaction
Vmax	Maximal velocity
Vs	Versus

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1. INTRODUCTION

The term β (1,4) endoglucanase or cellulase refers to the enzymes, which hydrolyse β 1, 4 - glucosidic linkages between anhydro glucose units in cellulose. They are mainly produced by fungi, bacteria and other cellulolytic organisms.

1.1 Cellulases

1.1.1 Classification of cellulases:

The enzymes that act on cellulose and derived products of cellulose can be divided into four groups (Coughlan, 1990).

(a) Endoglucanases [1,4(1,3; 1,4)- β-D-glucan 4-glucanohydrolase; EC 3.2.1.4]: They are inactive against crystalline celluloses such as cotton and Avicel (microcrystalline cellulose), but they hydrolyze amorphous celluloses (including amorphous regions of crystalline celluloses) and soluble substrates such as carboxymethylcellulose and hydroxyethylcellulose. Endoglucanase acts by hydrolyzing the β-glucosidic bond. The viscosity of the solution decreases with increase in reducing groups. Glucose, cellobiose, and cellodextrins are the products formed.

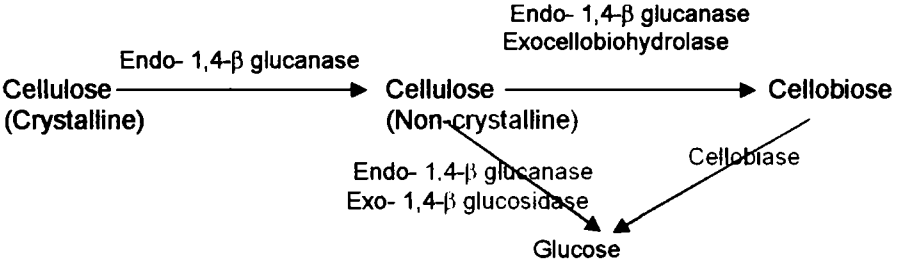
(b) Cellobiohydrolases (1,4- β-D glucan cellobiohydrolase, EC 3.2.1.91): They are exo-splitting enzymes i.e., they degrade amorphous cellulose by releasing cellobiose from the non-reducing ends of the substrate. When pure, they usually have little activity on cotton, but can degrade Avicel, microcrystalline cellulose, by as much as 40%. The rate of increase in reducing groups in relation to decrease in viscosity is much higher than for the endoglucanases.

Endoglucanases and cellobiohydrolases act synergistically in the hydrolysis of crystalline cellulose.

(c) Exoglucohydrolases (1,4- β-D-glucan glucobiohydrolase, EC 3.2.1.74): They hydrolyze the non-reducing ends of cellodextrins to glucose. The rate of hydrolysis decreases as the chain length of the substrate decreases.

(d) β-glucosidases (β-D- glucoside glucohydrolase, EC3.2.1.21): They cleave cellobiose to glucose and remove glucose from non-reducing end of small cellodextrins. The rate of reaction of β-glucosidase increases as the size of the substrate decreases; with cellobiose being hydrolyzed the fastest.

Hydrolysis of cellulose to glucose



1.1.2 Catalysis

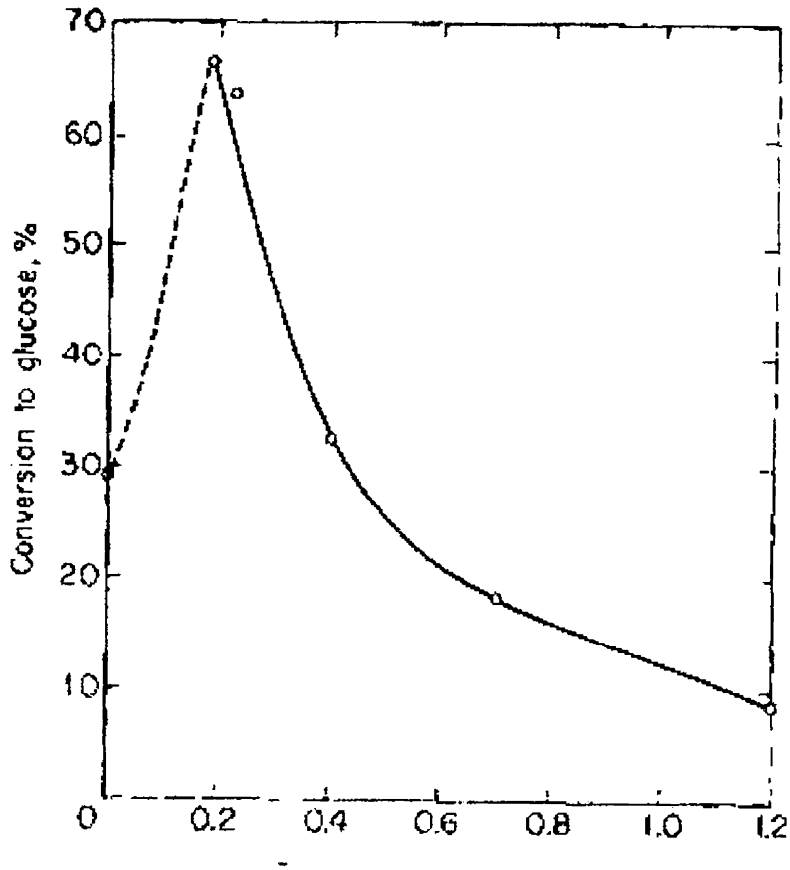
Endoglucanase comes under the family of glycoside hydrolases (EC 3.2.1.-), the enzymes that hydrolyse the glycosidic bonds in oligo and polysaccharides and hence share the same catalytic mechanism. Enzymatic hydrolysis of the glycosidic bond takes place via general acid catalysis requiring proton donor and a nucleophile/base. Hydrolysis of bonds in the substrate is either by inversion or by retention of the anomeric configuration of the β glycosidic bond in the substrate. For each family listed in the "CAZy – Carbohydrate Active enzymes" database, the catalytic mechanism, is well known as well as the type of

aminoacid residues acting as a nucleophile / base and as a proton donor are documented (Davies & Henrissat, 1995). The inverting enzymes use a single displacement mechanism. The attacking nucleophilic water molecule inverts the configuration at the anomeric center during hydrolysis. The retaining enzymes instead utilize a double displacement mechanism, containing a covalent glucosyl–enzyme intermediate where the nucleophile in the second step can be either a water molecule or another carbohydrate molecule (Sinnott, 1991; Mc Carter & Withers, 1994). These enzymes often have transglycosylating abilities. Based on the hydrophobic cluster analysis of the amino acid sequence of the catalytic domain, cellulases have been placed into 11 families (Henrissat, 1991; Henrissat & Bairoch, 1993). Typically cellulases have a catalytic domain and a cellulose-binding domain that are connected by a Proline/ Threonine/ Serine – rich peptide linker (Gilkes et al., 1991). The absence of cellulose binding domain, and associated peptide linker, has been observed in some endoglucanases and this appears to affect their substrate specificity but not their enzyme activity (Gilkes et al., 1991; Wang & Jones, 1995 a, b). The action of cellulases of *Aspergillus niger* on CMC with intermediate degrees of substitution is shown in Figure 1.1 (Cayle, 1962)

1.1.3 Application of cellulases

Cellulases have wide applications in food industry, paper and pulp industry, wood preservation, alcohol industry and pharmaceutical industry. Other uses of cellulase exist in waste water treatment, baking, preparation of animal feed, malting and brewing, extraction of food and vegetable juices (Béguin & Aubert,

Figure 1.1 Action of the cellulolytic enzyme complex of *Aspergillus niger* on carboxymethyl cellulose



Concentration of substrate 5%, Concentration of enzyme 0.8%, pH 5, temperature 37°C, time of hydrolysis 24 hr. (Cayle, 1962)

1994; Godfrey & West, 1996). The primary use of cellulase is digestive aid, particularly in animals. In the food industry, cellulase is used in the production of glucose syrups and coffee whiteners (Robinson, 1987). Cellulase in combination with other enzymes has been used in the production of protoplasts (Chen & Chiang, 1994; Coury et al., 1991) and in yeast cell lysis (Scott & Schekman, 1980). Cellulases can convert cellulosic waste to glucose, which can be subsequently converted to ethanol or other products of industrial applications (Béguin & Aubert, 1994). Cellulases have been used to increase the flavour of wine (Pérez-González et al., 1993; Shoseyov et al., 1990). Cellulases have also been used in detergents as stain remover, fabric softener, anti-redepositor and colour care agents. In the textile industry, they have been employed for stone washing, bio-polishing and for smooth surfacing of cotton fabrics (Rehm & Reed, 1999). Cellulase has considerable potential for utilizing cellulose waste material accumulated by the pulp and paper industry. This could be hydrolysed to glucose (Anderson, 2000). Immobilized biocatalysts are used in the production of medicines, chemicals, food and beverages and wastewater treatments (Abdel-Fattah et al., 1997).

1.2 The substrate

Cellulose: Cellulose, the most abundant natural polymer in the biosphere is composed of long chains of D-glucose molecules linked in a β (1,4) configuration. The glucose chains in cellulose are arranged in a manner that permits them to pack together in a crystal-like structure that is impervious to water. The density and complexity of cellulose makes it very resistant to hydrolysis without

preliminary chemical or mechanical degradation or swelling. It is the principal structural polysaccharide providing rigidity to plant cell walls, where it is usually associated with other polysaccharides such as xylan, lignin, hemicellulose, pectin and some proteins. It is therefore not perfectly crystalline but contains areas of more or less ordered structure, chain ends and dislocations (Chanzy, 1990). The percentage of cellulose varies from one cell to another and with growth. Cellulolytic turnover by living organisms has been estimated to be approximately 10^9 tons/year (Coughlan, 1985). The enzymatic degradation of cellulose is performed by a wide variety of plants, bacteria and fungi which possess endoglucanases, cellobiohydrolases and β- glucosidases acting in a synergistic fashion to facilitate complete cleavage of cellulose β – (1,4)- glycosidic bonds (Béguin and Aubert, 1994; Henrissat, 1994; Wood, 1992).

Carboxymethylcellulose prepared by reacting cellulose with chloroacetic acid and alkali, resulting in carbons 2, 3, and 6 of the glucose to carry carboxymethyl groups is the model substrate for detecting β(1,4)- endoglucanases. The water-soluble nature of carboxymethylcellulose provides good kinetic data in the assay for endoglucanase. The action of cellulase on carboxymethylcellulose can be determined by following the decrease in viscosity or by determining the rate of formation of reducing groups.

1.3 Occurrence of endoglucanase/ cellulase in nature

Fungi and microbes are one of the best sources of cellulases and many works have been carried out in this aspect. Endoglucanase has been isolated from yeast (Oikawa et al., 1998), *Bacillus* (Tian & Wang, 1998; Singh & Kumar, 1998;

Hakamada et al., 1997; Mawadza et al., 2000), *Clostridium* (Ng & Zeikus, 1981), *Cellvibrio fulvus* (Berg, 1975), thermophilic anaerobic bacterium (Creuzet & Frixon, 1983), thermoacidophilic gram-positive bacterium *Alicyclobacillus acidocaldarius* (Eckert & Schneider, 2003) and several fungi (Pettersson, 1969; Yuan et al., 2001; Mandels & Reese, 1957; Reese, 1956; Reese & Mandels, 1971; Hirvonen & Papageorgiou, 2003; Marques et al., 2003; Almin et al., 1975; Shoemaker & Brown, 1978; Wood & Mc Crae, 1978; Yamane et al., 1970). Endoglucanase has been isolated from the gut of larvae of yellow spotted longicorn beetle *Fracothea hilaris* (Sugimura et al., 2003) from the hindgut of *Copotermes* termites (Watanabe et al., 2002) from the digestive system of a lower termite *Coptotermes formosanus* (Nakashima & Azuma, 2000). The filter feeding nature and the fact that green mussel consume large quantities of phytoplankton - a cellulose rich organism and that it has the greatest growth rate points to the possibility of the abundance of endoglucanase in green mussel. Till date no work has been reported on the isolation of endoglucanase from *Pernaviridis,* which grow on a cellulose rich diet.

1.4 Green mussel as a source of endoglucanase

1.4.1 *Perna viridis*

The green mussel, *Perna viridis* (Plate 1.1), is a cosmopolitan and sedentary marine animal of the class bivalvia distributed along the intertidal coasts of India and throughout the Indo-Pacific coastal zones (Kuriakose, 1980; Siddall, 1980). *Perna viridis* has the greatest growth rate among the mussels studied till date (Shafee, 1979). The high growth rate of the green mussel is related to high

Plate 1.1 *Perna viridis* L. (Green mussel)

Photo by Debi Ingrao

salinity and an abundance of phytoplankton (Chatterji et al., 1984). Seasonal variation of growth rate with the activity of digestive enzymes in the digestive gland and crystalline style of *Perna viridis* has been studied. The activities of digestive enzymes including amylase and cellulase in the digestive diverticula and crystalline style were reported to be high in May and July and lower in February and October. Faster growth rates obtained for *Perna viridis* in Hongkong's summer were considered to be result of enhanced feeding, high temperature and organic content (Wong & Cheung, 1997). *Perna viridis* is a filter-feeding organism that not only collects bacteria, protozoa, phytoplankton, detritus and dissolved organic materials for food but also filters out other contaminants in the process.

Perna viridis is commercially important and cultivated in large quantities because of rapid growth rate and high population densities (Rajagopal et al., 1998) and that reproduction can be induced throughout the year (Coeroli et al., 1984). Alagarswami (1980) has reported on the growth relation of larvae of *Perna viridis* with a mixture of algal cultures. Upwelling (a phenomenon in which oxygen deficient, colder and nutrient rich deeper water comes up) and production of phytoplankton has been correlated with mussel growth during farming (Eastern & Mahadevan, 1980). Perna *viridis* is one of the most extensively studied marine organisms. Apart from commercial importance as a food source it is one of the best mussel species to test for biopollution (Phillips, 1980, IOC, 1981), including chlorinated pesticides (Radhakrishnan et al., 1986).

1.4.2 Is cellulase synthesized by green mussel?

There was a general thinking that cellulose digestion cannot occur in animals because they lack suitable enzyme systems and that the animals rely on symbiotic gut microorganisms to hydrolyze cellulose. The symbiotic anaerobic microorganisms, including bacteria, fungi and protozoa inhabiting the rumen of ruminants namely cattle and sheep play a vital role in digestion of cellulose. Cellulolytic enzymes have been isolated from rumen fungi (Trinci et al., 1994; Wallace, 1994; Gilbert et al., 1992; Xue et al., 1992) and some of them are well characterized. It has been reported that there are digestive enzymes present in the gut of the tropical earthworm *Pontoscolex corethrurus* (Zhang et al., 1993) and these enzymes were capable of degrading starch, pullulan, microcrystalline cellulose, carboxymethylcellulose, mannan, glucomannan, galactomannan and lichenin. Production of endogenous cellulase components by wood feeding insects has been reported by Slaytor, (1992). Cellulase have been isolated and purified from several other higher organisms such as edible snail, *Helix pomatia* (Rebeyrotte et al., 1967) and the marine mollusk *Littorina brevicula* (Purchon, 1977; Kiesov, 1982) and are reported to be synthesized and secreted by these organisms. A molluscan endoglucanase gene from the blue mussel, *Mytilus edulis* has been cloned and sequenced (Xu et al., 2001). The presence of cellulase along with other digestive enzymes in the digestive gland of *Perna* viridis has been reported (Toe & Sabapathy, 1990).

1.4.3 Digestive system of green mussel (*Perna viridis)*

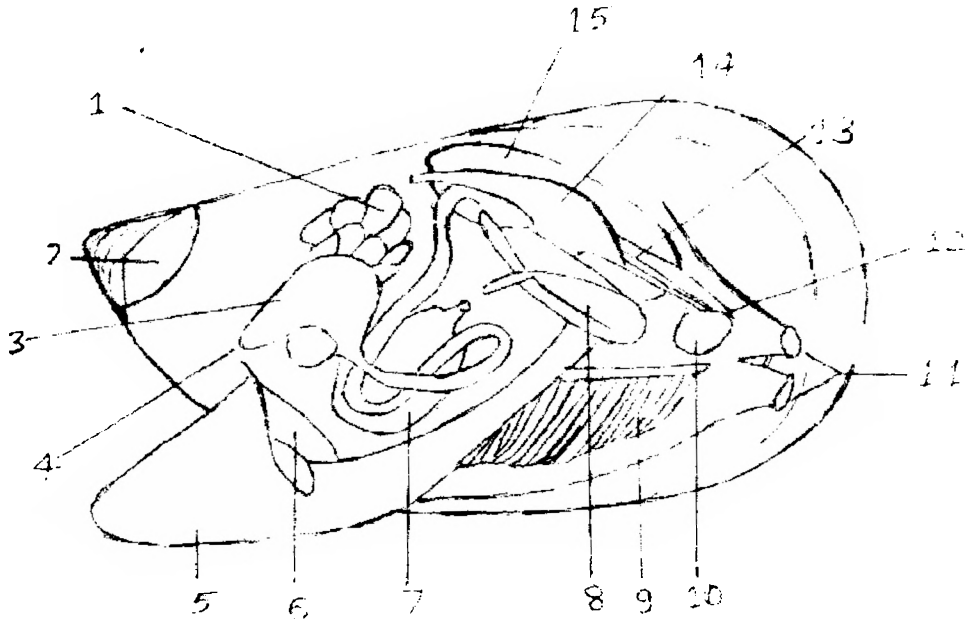
The mouth is covered by the ventral and dorsal side of the labial palps, and opens below the mid-intestinal gland. The oesophagus, which follows the mouth,

enters the mid-intestinal gland and leads to the stomach. Figure 1.2 shows the digestive system of green mussel. In the stomach a long style sac is present. It contains a crystaline style composed of solidified mucus and enzymes. When the style rotates, it reels in food laden with mucus. The rotation of crystalline style helps the digestive processes in green mussel. The style projects into the stomach and is worn away liberating digestive enzymes such as α amylase, cellulase, laminarinase, chitinase, α-glucosidases, β-fructosidase, arbutinase, α-D mannosidase, trypsin, leucine aminopeptidase, aminotripeptidase, aryl esterase, lipase (Alemany et al., 1973; Birkbeck et al., 1984; Onishi et al., 1985; Kaur, 1977).

1.5 Present Investigation

1) To separate endoglucanase by preliminary purification methods form the homogenized digestive gland of *Perna viridis*.
2) To further purify the endoglucanase on Sephadex G100 column by elution with 50mM imidazole and 50mM EDTA buffers.
3) To test the purity of endoglucanase by polyacrylamide gel electrophoresis.
4) To determine the molecular weight of endoglucanase by SDS PAGE.
5) To analyse the amino acid composition of endoglucanase by HPLC.
6) To measure the kinetic properties of endoglucanase.
7) To evaluate the effect of certain ions on the activity of endoglucanase
8) To immobilize the enzyme on polyacrylamide gel and chitosan.

Figure 1.2 Digestive system of Green mussel (*Perna viridis*)



1. Digestive gland
2. Anterior Adductor Muscle
3. Stomach
4. Mouth
5. Foot
6. Labial Palps
7. Gonad
8. Kidney
9. Gill
10. Posterior Adductor Muscle
11. Siphons
12. Anus
13. Intestine
14. Ventricle
15. Pericardium

2. REVIEW OF LITERATURE

The Report of the Commission on Enzymes of the International Union of Biochemistry (1961) termed "cellulase" as the trivial name for β-(1,4)-glucan-4-glucanohydrolases, i.e., for enzymes which hydrolyse β-1,4-glucans at linkages which are not restricted to terminal linkages. Cellulase was earlier reviewed by Emert et al., (1974) and Whitaker (1971). Hydrolysis and utilization of cellulose to provide energy to the organism was thought to be carried out exclusively by microorganism. But now it appears that some animal species, including termites and cray fish produce their own indigenous microflora (Watanabe & Tokuda, 2001). Li et al., (Report, 1961) mention that their endoglucanase can be coded according to the Commission on Enzymes of the International Union of Biochemistry as a β-(1,4)-D-glucanohydrolase (E.C.3.2.1.4). It indicates the substrate, as a β-(1,4) polymer of D-glucose, which is split by hydrolysis of β-(1,4)-glucosidic linkages at random attack, not confined to the terminal ends.

2.1 Cellulose

Plants have high cellulose content, approximately 35 to 50% of plant dry weight (Lynd et al., 1999). Cellulose is present in pure state in cotton balls. In most cases, cellulose fibres are embedded in a matrix of other structural biopolymers, primarily hemicelluloses and lignin, which comprise 20 to 35 and 5 to 30 % of plant dry weight (Lynd et al., 1999; Marchessault & Sundararajan, 1993; Van Soest, 1994). Although these matrix interactions vary with plant cell type and with maturity (Wilson, 1993), they are a dominant structural feature limiting the

rate and extent of utilization of whole, untreated biomass materials. Cellulose is synthesized in nature as individual molecules (linear chains of glucosyl residues), which undergo self-assembly at the site of biosynthesis (Brown & Saxena, 2000). Approximately 30 individual cellulose molecules are assembled into large units known as elementary fibrils (protofibrils), which are packed into larger units called microfibrils, and these are in turn assembled into the familiar cellulose fibres. Regardless of orientation of adjacent chains, they are stiffened by both intra-chain and inter-chain hydrogen bonds. Adjacent sheets overlie one another and are held together (in Cellulose I, the most abundant form of cellulose in nature) by weak intersheet van der waals forces. Despite the weakness of these interactions, their total effect over the many residues in the elementary fibril is considerable (Pizzi & Eaton, 1985). The crystalline nature of cellulose implies a structural order in which all of the atoms are fixed in discrete positions with respect to one another.

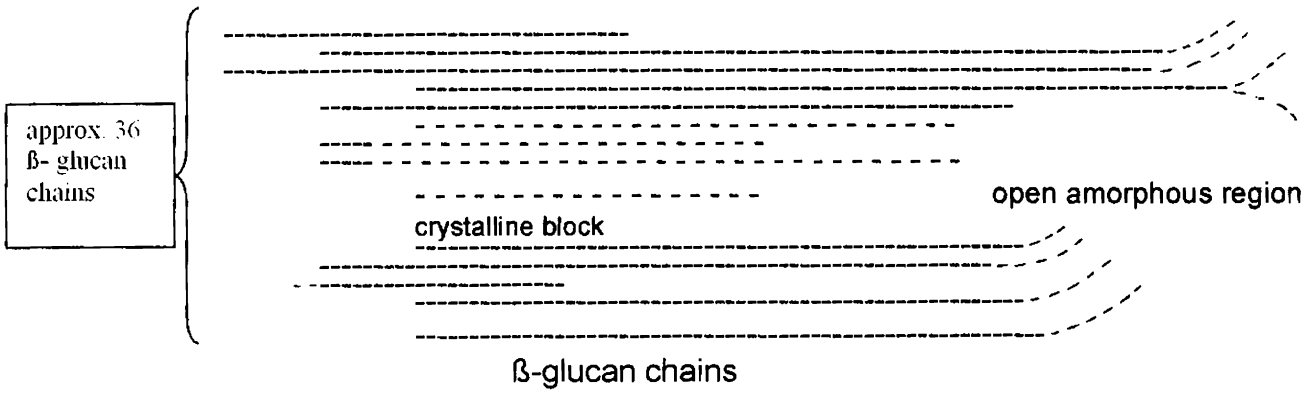
Although cellulose forms a distinct crystalline structure, cellulose fibres in nature are not purely crystalline. The degree of crystallinity is variable. Distribution of crystallinity is "lateral" which portrays a population of cellulose fibres in statistical terms as a continuum from purely crystalline to purely amorphous, with all degrees of order in between (Marchessault & Howsmon, 1957). Apart from crystalline and amorphous regions, cellulase fibres contain various types of irregularities, such as twists of the microfibrils, or voids such as surface micropores, large pits and capillaries (Blouin et al., 1970; Cowling, 1975; Fan et al., 1980a; Marchessault & Sundararajan, 1993). Schematic representation of a

cellulose fibre is shown in Figure 2.1. The fibres are hydrated by water when immersed in aqueous media and some micropores and capillaries are sufficiently spacious to permit penetration by cellulolytic enzymes (Stone et al., 1968). Purified celluloses used for studies of hydrolysis and microbial utilization vary considerably in fine structure. The choice of substrate thereby affects the results obtained. Microcrystalline celluloses (e.g. Avicel and Sigma cell) are nearly pure cellulose, and the dilute acid treatment used in their preparation removes both hemicelluloses and the more extensive amorphous regions of the cellulose fibres. Like plant cellulose, bacterial cellulose is highly crystalline, but the two celluloses differ in the arrangement of glucosyl units within the unit cells of the crystallites (Atalla & Vanderhart, 1984) and genetic evidence suggests that the two celluloses are synthesized by enzymatic machinery that differs considerably at the molecular level (Brown & Saxena, 2000). The two celluloses also differ substantially in rate of hydrolysis by fungal cellulases (Hayashi et al., 1997) and in rate of utilization by mixed ruminal bacteria (Schofield et al., 1994; Weimer et al., 2000). The variable structural complexity of pure cellulose and the difficulty of working with insoluble substrates have led to the wide use of the highly soluble cellulose ether, carboxymethylcellulose (CMC), as a substrate for studies of endoglucanase production (Lynd et al., 2002).

2.2 Glycoside hydrolase families

Enzymes are designated according to their substrate specificity, based on the guidelines of the International Union of Biochemistry and Molecular Biology. The cellulases are grouped with many of the hemicellulases and other

Figure 2.1: Schematic representation of a cellulose fibre (Robinson, 1987)

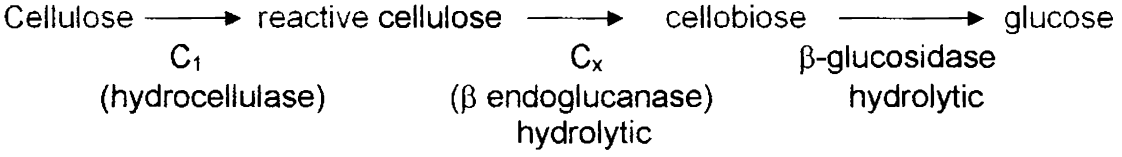


polysaccharides as O-glycoside hydrolases (EC.3.2.1.x). With several thousand glycoside hydrolases being identified, alternative classifications into families were suggested based on aminoacid similarity (Henrissat, 1991). This classification has been updated several times (Henrissat & Bairoch, 1993, 1996), but with the exponential growth in the number of glycoside hydrolases identified, Coutinho & Henrissat (1999) have begun to maintain and update the classification of glycoside hydrolases families at the Expasy server (http://afmb.cnrs-mrs.fr/~pedro/CAZy/db.html). Families were defined based on aminoacid sequence similarities. There is usually a direct relationship between the aminoacid sequence and the folding of an enzyme, and as the tertiary structures of many proteins were added, it became clear the families contain basic enzyme folds (Henrissat et al., 1995). Classification into families defines the modules of such enzymes and resolves the contradiction about substrate specificity for multifunctional enzyme. The family classification also sheds light on the evolution of glycoside hydrolases. Some families contain enzymes with different substrate specificity e.g. family 5 contains cellulases, xylanases and mannanases. This suggests divergent evolution of a basic fold at the active site to accommodate different substrates. At the same time cellulases are found in several different families [families, 5, 6, 7, 8, 9, 10, 12, 44, 45, 48, 61 and 74] suggesting convergent evolution of different folds resulting in the same substrate specificity. Family 9 contains cellulases of bacteria, fungi and plants (Del Campillo, 1999) and animals (protozoa & termites) (Watanabe & Tokuda, 2001). In contrast family 7 contains only fungal hydrolases and family 8 contains only

bacterial hydrolases. The classification of cellulases into families allows easy access to information on structure, mechanism and evolutionary origin. Henrissat et al., (1998) recently proposed a new nomenclature for hydrolases in which the first three letters designate the preferred substrate, the next digits designate the glycoside hydrolase family, and the following capital letters indicate the order in which the enzymes were first reported. For e.g., the three enzymes cellobiohycrolase I (CBH I), CBH II and endoglucanase I of *Trichoderma reesei* are designated Cel 7A (CBH I), Cel 6A (CBH II) and Cel 16B (EG I). However, this new nomenclature has not been completely accepted due to lack of substrate specificity, for instance the distinction between endoglucanase and CBH activity.

2.3 Mechanism of cellulase degradation by enzymes

Several models have been described to study the enzymatic mechanism by which cellulose is being hydrolyzed. Reese et al., (1950) had stated that many microorganisms are able to hydrolyze modified celluloses, but only some of them are able to attack native cellulose such as mature, dried cotton. They suggested that a C_1-C_x system should make the latter true cellulolytic organism capable of completely solubilizing native cellulose. Mandels and Reese (1964) have described their C_1-C_x concept in the following schematic way:



The hydrolytic C_x is a β-1,4-glucanase, able to attack all celluloses in the range from soluble cellulose derivatives to celluloses "swollen" by alkali or acids or by means of mechanical treatments such as grinding. When the substrate does not offer hindrance to the approach of the enzyme molecule, and the end effects of very short chains are absent, the enzyme is of a random-splitting type. Norkrans (1967) has reported that the endoglucanase is not specific to the bond being broken but to the reducing end unit being split off. Two species of *Trichoderma*, namely *Trichoderma viridae* and *Trichoderma koningii*, were found to give cell-free preparations capable of solubilizing cotton fiber (Mandels & Reese, 1964; Halliwell, 1965; Li et al., 1965). Halliwell obtained complete solubilization of cotton fibers with quantitative conversion to glucose within 19 days by a culture filtrate from *Trichoderma koningii*. In the first phase (7 days) short fiber fragments were produced and only minor quantities of soluble products. When soluble and insoluble products each constitute 40 to 50% of the weight of the initial substrate; the quantity of sugars increases at the expense of the insoluble fragments. Enzyme preparations from *Myrothecium verrucaria* could not solubilize the short fragments formed. Cellulose powder was solubilized by *Trichoderma* filtrate at the same rate as cotton fibers (19 years), hydrocellulose required almost four times that period.

According to the model of native cellulose as a fringe micelle, fragmentation of fibres has been interpreted as a chain shortening in the same way as enzymic attack by cellulase from *Penicillium variabile* (Norkrans, 1967). Amberlite XE-64 fractionated an enzyme preparation from *Penicillium variable* into a fraction with

activity towards carboxymethylcellulose and a fraction with activity towards carboxymethylcellulose and α-cellulose; the latter did not give cellobiose as a hydrolysis product. A fraction with activity towards carboxymethylcellulose but not towards swollen cellulose or powdered cellulose has also been reported to be obtained from *Aspergillus saitoi* (Jurášek, 1967). Mandels and Reese worked with *Trichoderma viridae*. After treating dewaxed cotton slivers for 45 days with culture filtrates from this organism, a weight loss of 60% was noted. This is 30 times or more the loss caused by *Myrothecium verrucaria* or *Chaetomium globosum*. Cotton was found to be the most resistant of the pure celluloses. Woody materials were also resistant unless they were thoroughly ground by ball milling. Filter paper or newspaper showed considerable breakdown in 1 day, thus 50g of newspaper gave rise to 12.8g of sugars, mainly glucose, but also cellobiose and xylose, when treated with *Trichoderma* filtrate. After repeated chromatograms on DEAE- dextrans, they succeeded in separating components into C_1, C_x and β-glucosidase activity. They assumed C_1 "to act in a way to permit an increased moisture uptake, hydrating the cellulose and pushing apart the closely packed chains", to make the linkages accessible for the action of C_x (β-1, 4 - endoglucanase). Li et al., (1965) maintain that the key property of the C_1 component is its capacity to attack highly crystalline cellulose. Consequently, they used crystalline aggregates of hydrocellulose, or the commercially available Avicel, which constitutes an easily handled "eucellulosic" substrate, for testing a cellulase system. The starting enzyme was obtained from wheat bran-sawdust cultures of *Trichoderma viridae*. They have been able to separate this crude

preparation into different components with distinct enzymic properties. Although none of the components alone could account for the enzymic overall process of the crude preparation, appropriate combinations did. The components are hydrocellulase, endoglucanase and exoglucanase (Norkrans, 1967).

Hydrocellulase correspond to the C_1 component, being the only one having the capacity to attack crystalline cellulose at any appreciable rate. The primary product of attack is cellobiose. At the first step of purification, on Avicel column, irrigated by sodium citrate buffer of pH 4.8, the hydrocellulase was completely retained, contrary to all the other components (Norkrans, 1967). This component showed an approximate molecular weight of 60,000. Endoglucanase has a minimum molecular weight of 26,000 (based on amino acid composition, one methionine per molecule) and a most probable molecular weight of 52,000 according to sedimentation data. It was relatively thermo-stable and it behaved just as a typical C_x - enzyme. The optimum length of its substrate chain was atleast 6 glucosyl unit according to K_m values derived from hydrolysis of glucomers varying from cellobiose through cellohexose. By using terminally substituted sugars (cellotetrosyl- and cellopentosyl sorbitol), higher rates of hydrolysis for interior linkages could be shown (Whitaker, 1954). Exoglucanase has a molecular weight of 76,000, approximated form sedimentation data. All of the thirteen β-glucosides tested were hydrolysed.

The difficulties in studying enzymatic hydrolysis of cellulosic materials are attributable to the complex property of cellulose and its constituents as well as to

the multiplicity and complexity of the cellulase system (Lee et al., 1982; Ryu & Mandels, 1980). Various kinetic models have been developed to elucidate the mechanism of hydrolysis of cellulase. One model is based on the structural features of the substrate (Fan et al., 1980b; Grethlein, 1985) while other based on properties of the cellulase enzyme and the mass transfer in reaction system. Kinetic model of the full time course of hydrolysis including enzyme adsorption has been developed by (Lee & Fan, 1983; Converse et al., 1988, Converse, 1993). A kinetic study of the hydrolysis of celluloses by Endoglucanase I and Exoglucanase II purified from *Trichoderma viridae* cellulose was performed (Kim et al., 1995). It was found that a more reactive endoglucanase acts randomly, mainly on amorphous or modified cellulose such as carboxymethylcellulose to produce glucose. The exoglucanase removes a cellobiose unit from the non-reducing end of the cellulose chain. This compound attacks neither the amorphous or modified cellulose nor the crystalline cellulose to any significant extent (Kim et al., 1994).

Earlier studies reported on the production of cellulases by microorganisms includes: bacteria: *Sporocytophaga myxococcoides*, *Ruminococcus albus*, and a thermophilic bacterium; a streptomycete: Streptomyces antibioticus (Enger & Sleeper, 1965); molds: *Rhizopus* spp. various species of Penicillium, Trichoderma, and Aspergillus terreus and Penicillium variable (Pal and Ghosh, 1965), a thermophilic strain of *Aspergillus fumigatus*, *Pyrenochaeta terrestris* (Horton & Keen, 1966); Mycorrhizal fungi and higher fungi: *Fomes annosus*. Various cellulase-producing microorganisms are listed in Table 2.1. The cellulase

Table 2.1 List of various cellulase producing microorganisms

Fungi

Acremonium cellulolyticus
Aspergillus acculeatus
Aspergillus fumigatus
Aspergillus niger
Asperillus oryzae
Chrysosporium lignorum
Chrysosporium lucknowense
Fusarium solani
Humicola insolens
Irpex lacteus
Melanocarpus albomyces
Merulius lacrymans
Mucor circinnelloides
Myrothecium verrucaria
Neisseria sicca
Orpinomyces joyonii
Penicillium citrinum
Penicillium funmiculosum
Penicillium notatum
Penicillium variable
Pervotella spp.
Phanerochaete
Piromyces equi
Rhizopus delmar
Rhizopus oryzae
Schizophyllum commune
Sclerotinia sclerotiorum
Sclerotium rolfsii
Sporotrichum cellulophilum
Sporotrichum dimorphosporum
Talaromyces emersonii
Thermoascus aurantiacus
Thermoascus longibrachiatum
Thielavia terrestris
Trichoderma koningii
Trichoderma reesei
Trichoderma viridae
Volvariella volvaceae

Bacteria

Alicyclobacillus acidocaldarius
Bacillus spp.
Bacteroides succinogens
Cellulomonas
Cellvibrio fulvus
Clostridium acetobutylicum
Clostridium thermocellum
Pervotella spp.
Rhodothermus
Ruminococcus albus
Streptomyces sp.
Thermobifida fusca

Actinomycetes

Rhodothermus
Streptomyces sp.
Thermoactinomyces sp.
Thermomonospora curvata

in the digestive juice of the edible snail, *Helix pomatia*, is prominent in the early literature on cellulases but its origin has been a matter of some controversy. Later it was suggested to be produced by the snail itself and not by its microflora (Jurášek, 1967).

For the purpose of ease and lucidity the findings on cellulases are classified based on the source from which it is obtained.

2.4 Fungi as a source of Endoglucanase

Fungi endoglucanase forms nearly one third of the research work carried out with endoglucanase/cellulases. Consequently the work done on fungi endoglucanase forms the basis and model for studies of other endoglucanase. Detailed reviews on fungi Endoglucanases have been published (Lynd et al., 2002; Norkrans, 1967). Amino acid composition indicates a molecular weight of 31,000. The ability of fungi to digest cellulose has been reported (Bucht & Eriksson, 1969; Keilich et al., 1969). Fungi are the well-known agents of decomposition of organic matter in general and of cellulosic substrates in particular (Carlile & Watkinson, 1997; Montegut et al., 1991). A number of species of the anaerobic *Chytridomycetes*, the primitive group of fungi, are well known for their ability to degrade cellulose in the gastrointestinal tracts of ruminant animals. Aerobic fungi are also capable of cellulolysis. Within the approximately 700 species of zygomycetes only certain members of the genus *Mucor* have been shown to possess significant cellulolytic activity. They are better known for their ability to utilize soluble substrates. The much more diverse subdivisions Ascomycetes,

Basidiomycetes and Deuteromycetes (Carlile & Watkinson, 1997) contain large numbers of cellulolytic species. *Bulgaria, Chaetomium* and *Helotium* (Ascomycetes); *Coriolus*, *Phanerochaete*, *Poria*, *Schizophyllum* and *Serpula* (Basidiomycetes) and *Aspergillus*, *Cladosporium*, *Fusarium*, *Geotrichum*, *Myrothecium*, *Paecilomyces*, *Penicillium* and *Trichoderma* (Deuteromycetes) are some of the genera that have received considerable study with respect to their cellulolytic enzymes and/or wood- degrading capability.

Carboxymethylcellulase has been identified in rotting fungi (Bucht & Ericksson, 1969; Keilich et al., 1969). Cowling (1975), studying wood decay, makes the following statement about the white-rotting *Polyporus versicolor*, "the organisms degraded the crystalline and amorphous cellulose simultaneously".

Several isoenzymes of cellulase have been studied. Table 2.2 lists the works carried out and the number of isoenzymes of cellulases identified in each of these organisms.

Selby et al., (1963) suggested that the cellulase system of *Myrothecium verrucaria* contained at least two enzyme types that differed in the rate and extent of their attack on fibrous cellulose such as cotton yarn. It was suggested that the A enzyme necessary for extensive degradation of cotton was present in small amounts only in the culture filtrate as normally prepared, and that it was "exhausted" by exposure to cotton yarn. Another cellulase, the B enzyme, was not so deactivated, but was able to weaken cotton to a limited extent only. The B activity was measured as carboxymethylcellulase activity, the A activity as loss of

Table 2.2 List of microorganism that produce cellulase - isoenzymes.

Microorganisms	No. of Isoenzymes	References
Myrothecium verrucaria	2	Selby et al., 1963
Myrothecium verrucaria	3	Selby & Maitland, 1965
Chrysosporium lignorum	5	Erriksson & Pettersson, 1975 Almin et al., 1975 Erriksson & Rzedowski, 1989 a, b
Rhizopus oryzae	2	Murashima et al., 2002
Thermoascus aurantiacus	3	Tong et al., 1980
Polyporus vesicolor	2	Pettersson & Porath, 1963
Trichoderma reesei	2	Bhikhabhai et al., 1984
Humicola insolens	7	Schülein, 1997
Penicillium citrinum	11	Olutiola, 1976
Trichoderma viridae	2	Okada, 1975
Trichoderma koningii	2	Iwasaki et al., 1964
rotinia sclerotiorum	2	Waksman, 1991
ilvus	5	Enger & Sleeper, 1965
	7	Chaudhary et al., 1997
	6	Irwin et al., 1993

tensile strength in cotton yarn. When *Myrothecium verrucaria* filtrates have been fractioned by gel filtration on Sephadex G-75 (Selby & Maitland, 1965), three major cellulolytic components were obtained. The middle component (II) with a molecular weight of about 30,000 represented 90% of the total carboxymethylcellulase activity and thus seemed to contain the B enzyme. Reruns after exposure to cotton showed that it only slightly affected this activity. The other two components (I and III), having molecular weights of about 55,000 and 5300, respectively, were maintained responsible for the activity of the filtrate toward cotton, and were removed or deactivated by exposure to it, behaving as an A enzyme in these respects. These observations accord with the previously reported behavior of the whole culture filtrate.

In the previous publication (Selby et al., 1963), it was suggested that the loss of A could result in genesis of B, possibly for the combination of A with the products of cellulolysis. No evidence of such inter-conversions was found. Thus if A activity is lost by the formation of a soluble enzyme-carbohydrate complex, the change in molecular weight was too small to be detected by gel-filtration on Sephadex G-75. C_1 component is essential for the extensive degradation of cotton, but is without significant action of its own, either on cotton, carboxymethylcellulose, or cellobiose. It acts synergistically with C_x. The small solubilizing power of C_1 acting alone is enhanced when the incubation with cotton takes place on a dialysis membrane (Norkrans, 1967).

Five endoglucanases of *Chrysosporium lignorum* have been characterized and reported (Almin et al., 1975; Eriksson and Pettersson, 1975 and Erriksson and Rzedowski, 1960 a,b). A cellulolytic enzyme has been purified from *Penicillium notatum* by tannin precipitation, extraction with pyridine acetate buffer, IEC, 80% ammonium sulphite precipitation and Sephadex G75 chromatography (Pettersson & Porath, 1966). The purification procedure of this cellulase is shown in Table 2.3. The enzyme was active towards carboxy methylcellulose, hydroxyethylcellulose and ethylhydroxyethylcellulose, cellodextrins. An endoglucanase was isolated from culture of fungus *Mucor circinelloides* (NRRL 26519) and properties studied (Saha, 2003). Two extracellular endoglucanases produced by *Rhizopus oryzae*, *isolated* from the soil, had been purified and characterized under family 45 endoglucanases (Murashima et al., 2002).

Cellulases from aerobic fungi have been extensively studied than those of any other physiological group and fungal cellulases currently dominate the industrial applications of cellulases (Gusakov et al., 1992; Nieves et al., 1998; Sheehan & Himmel, 1999). In particular, the cellulase system of *Trichoderma reesei* (teleomorph: *Hypocrea jecorina*, intitially called *Trichoderma viridae*) has been the focus of research for 50 years (Mandels & Reese, 1957; Reese, 1956; Reese & Mandels, 1971, Reese et al., 1950). It is complete in that it can convert native cellulose as well as derived celluloses to glucose (King & Vessal, 1969). Cellulase I and III isolated from *Thermoascus aurantiacus* were active towards CMC (Tong et al., 1980). CMC has been used as effective substrate for

Table 2.3 Purification procedure of cellulase from Penicillium notatum (Pettersson & Porath, 1966)

Fraction	Volume (ml)	A_{280}	Total A_{280}	Cellulase activity per ml (U)	Total cellulase activity (U)	Specific activity (U/mg)	Recovery of main material	Total recovery (%)
Crude extract	692	217	150000	33.0	22800	0.152	100	100
Chromatography on DEAE Sephadex A-25	1720	13.2	22700	9.11	15700	0.690	69	88
(NH4)2SO4 (80%)	74	56	4140	197	14600	3.51	64	100
Gel filtration on Sephadex G-75 & concentration	8	146	1168	1280	19200	8.77	45	86
Electrophoresis & concentration	5.5	164	902	1760	9680	10.7	42	-

endoglucanase (Chernoglazov et al., 1983; Lin & Stutzenberger, 1995; Lucas et al., 2001)

A 23 KDa endo-1, 4-β- glucanase (Cel 12 A), a wall hydrolytic enzyme from *Trichoderma reesei* with potent ability to induce extension of heat – inactivated type I cell walls have been identified and studied. This endoglucanase (Cel 12A) belong to glycoside hydrolase family 12. Extension of heat inactivated walls from Cucumber (*Cucumis sativus*) hypocotyls was induced by Cel 12A after a distinct lag time and was accompanied by a large increase in wall plasticity and elasticity (Yuan et al., 2001). A thermophilic fungus *Melanocarpus albomyces* was reported to produce a 20 KDa endoglucanase found particularly useful in textile industry. Its crystal structure has been determined (Hirvonen & Papageorgiou, 2003). Its crystal structure proves it to be classified under glycoside hydrolase family 45.

The reports on *Trichoderma viridae* cellulase are numerous and varied (Li et al., 1965; Kim et al., 1994; Shoemaker & Brown, 1978). Li et al., (1965) have reported fractionation of a commercial enzyme preparation into (a) a "hydrocellulase" fraction with high activity toward Avicel, (b) an "endoglucanase" fraction with high activity toward carboxymethylcellulose (and the soluble oligoglucosides, including cellobiose) and (c) an "exoglucanase" fraction which, unlike the endoglucanse, degraded the soluble oligoglucosides by cleaving glucose units from the nonreducing end. The separations depended on (1) adsorption of the "hydrocellulase" on Avicel in the presence of citrate buffer

(and desorption by water) and (2) adsorption of the "endoglucanase" on alkali-swollen cellulose in the presence of phosphate buffer (and desorption by water). The molecular weight of the "hydrocellulase" was estimated from its sedimentation rate to be about 60,000, that of the "endoglucanase" to be about 50,000 and that of the "exoglucanase" to be about 76,000.

Mandels and Reese (1964) reported a partial separation of activity toward cotton linters and activity toward carboxymethylcellulose by a fractionation on DEAE-Sephadex (A-50). Chromatography on powdered cellulose partially separated activity towards cotton fibres, filter paper and carboxymethylcellulose. Chromatography on a gauze column separated the fraction with high activity toward cotton into two fractions with different activities towards vegetable cell walls. Three to four fractions with varying activities towards filter paper and carboxymethylcellulose was obtained by electrophoresis in a block of starch grains. Chromatography of the crude enzyme on Amberlite XE-64 gave three fractions with activity toward cotton, filter paper, and carboxymethylcellulose; the major fraction on refractionation on DEAE-Sephadex (A - 50) gave five fractions with activity towards the above substrates and towards soluble oligoglucosides including cellobiose (Jurášek, 1967).

Extensive purification of *Myrothecium verrucaria* cellulase enabled Whitaker et al., (1954) to state that the cellulase was an ellipsoid-shaped protein, approximately 50 x 200 A° in size with a molecular weight of 63,000; by later improved procedures this was amended to 49,000 (Datta et al., 1963). Molecular

weights as low as about 10,000 have been found. Selby and Maitland (1965) even mentioned 5300 for component III, an estimation that however, might be quite preliminary as it is only deduced from a Sephadex column, calibrated with proteins and substances of known molecular weights in a range from 670,000 to 255. Even a molecular weight of about 5000, presumably corresponding to protein with a molecular diameter not smaller than 15 A° in diameter, seems to be too large atleast for action within crystalline cellulose. Pores of about 100A° in diameter, communicating with pores of 10A°, have been demonstrated in native cellulose (Frey-Wyssling, 1959). Two cellulases were reported to be purified from the culture filtrates of *Polyporus versicolor*. The enzyme was concentrated by dry Sephadex G-25 and fractionated on Sephadex G-75; one fraction (D) when refractionated on Sephades G-75, gave an apparently homogeneous enzyme (B1) (Jurášek, 1967).

Cellulase was separated from culture filtrates of *Myrothecium verrucaria* by two methods. In the first method enzyme was precipitated by ammonium sulfate and freed of noncellulase impurities by elution from Sephadex G-75, precipitation with polymethacrylic acid, and displacement from Amberlite CG-50 with a gradient of urea in citrate buffer. In the second method enzyme was precipitated with ammonium sulfate and freed of noncellulase impurities by precipitation with polymethacrylic acid, elution from DEAE cellulose with 7M urea-phosphate buffer and elution form Sephadex G-75. The two methods gave products with identical specific activities towards CMC. The use of buffered 7 M urea for the elution from DEAE cellulose required comment. It was used to prevent enzymic attack

on the resin; if this was not prevented, the enzyme became complexed with DEAE substituted oligoglucosides and there upon became subject to proteolysis; the consequent fall in molecular weight (e.g., from 50,000 to 7,000) was accompanied by loss of activity towards CMC (Whitaker et al., 1963; Datta et al., 1963). Chromatography on calcium phosphate separated two components with activity toward carboxymethylcellulose from a commercial *Aspergillus niger* enzyme preparation; treatment with laminarin removed the trace β(1 → 3) glucan activity (Jurášek, 1967).

An endoglucanase from *Thermomonospora curvata* was purified to electrophoretic homogeneity by ammonium sulphate precipitation, ion exchange chromatography, size exclusion HPLC (Lin & Stutzenberger, 1995). Two endoglucanases from *Trichoderma reesei* strain QM 9414 was purified by Bhikhabhai et al., 1984). Study was carried out by adding peptide tag (Trp-Pro-Trp-Pro) to endoglucanase I of *Trichoderma reesei* to change the partitioning in aqueous two-phase system comprising of thermo-separating ethylene oxide (EO) – propylene oxide (PO) random copolymer EO-PO (50:50) (EO50PO50) and dextran. The fusion position at the end of cellulose binding domain, with the spacer Pro-Gly was shown to be optimal (Collen, 2001). Endoglucanase of *T. reesei* have been reported as industrially important. A fusion protein EgI (Cel 7B) (EG I - hydrophobin I) from *T. reesei* culture filtrate has been extracted and purified in a one step PEG- sodium/ potassium phosphate two phase aqueous system (Collen et al., 2002). The cellulase system of the thermophilic fungus *Humicola insolens* has been found to possess a group of enzymes that allows

the efficient utilization of cellulose (Table 2.2). The *H. insolens* cellulose system is homologous to the *T. reesei* system and also contains atleast seven cellulases; two cellobiohydrolases [CBH I & CBH II] and five endoglucanases [Endoglucanase I, Endoglucanase II, Endoglucanase III, Endoglucanase V, Endoglucanase VI] (Schülein 1997). Tong et al., 1980 have purified three cellulases of molecular weight 79000 (Cel III), 49000 (Cel II) and 34000 (Cel III) from Thermophilic fungus *Thermoascus aurantiacus*. The effect of acetic acid and furfural on the cellulose production of a filamentous fungus *Trichoderma reesei RUT C30* was studied by Szengyel & Zacchi (2000). A low mol wt endoglucanase (13,000) has been purified from the cellulose complex of *Trichoderma koningii* and its properties studied (Churilova et al., 1980). The localization of cellulase in the hyphae of fungus *Achlya ambisexualis* was studied by (Nolan & Bal, 1974). Endoglucanase I from culture fluid of *Volvariella volvacea* was purified by ion exchange and gel filtration chromatography (Ding et al., 2002). Garcia et al., (2002) has resolved the catalytic domain from *T. reesei* using anion exchange chromatography. The pure fractions were analysed using lectins and electrospray MS. Isolated N-glycans were analysed by fluorophore assisted carbohydrate electrophoresis and amine adsorption HPLC. Shi et al., (2002) purified cellulose of *Aspergillus niger* using affinity membrane followed by anion exchange on POROS 20HQ.

Eriksson & Pettersson studied an electophoretically homogeneous β-endoglucanase from *Penicillium notatum*, having no activity against native cellulose. The molecular weight is 78,000 and the amino acid composition is known, giving

evidence for the presence of two half cysteines. The enzyme is composed of a single polypeptide chain, internally crosslinked by two cysteines as well as by chloride ions. The activity could be restored by cysteine residues in a disulfide bridge. The enzyme was strongly inhibited by mercuric ions. The activity could be restored by cysteine as well as by chloride ions. By electrolytic reduction the disulfide bridge, present in the enzyme, was shown to be essential for the activity. The β-endoglucanase was not attacked by exopeptidases under non denaturing conditions, indicating a solid structure; on the whole, cellulases are so strikingly stable to, e.g., pH and temperature, that it surprises veteran protein chemists, although the susceptibility of the enzyme increases, naturally enough, with purification. Treatment with endopeptidases caused a pronounced decrease in activity. Active fragments could not be obtained with endopeptidases. An electrophoretically and ultracentrifugally homogenous cellulase from *Myrothecium verrucaria*, active with respect to ground or swollen cellulose, carboxymethylcellulose, and a series of oligoglucosides (Whitaker et al., 1963) were found to have 14 cysteine residues.

From anaerobic fungus *Piromyces spp.* Strain E2 three dominant proteins was purified by cellulose affinity method consisting of steps of EDTA washing followed by elution with water (Steenbakkers et al., 2002). Studies have shown that isoenzymes of endoglucanase from *Trichoderma longibrachiatum* differed in their ability to be adsorbed on CMC, amorphous and crystalline celluloses. Moriyoshi et al., (2003) have studied the role of endoglucanase from *Neisseria sicca (SB)* in synergistic degradation of cellulose acetate. Its purification

procedure is shown in Table 2.4. They have isolated Endoglucanase I of 50 KDa mol wt, optimum pH 5-6 and temperature 45°C. It's K_m and V_{max} on CMC was determined to be 0.448% and 13.6 µmol/ min/ mg (Table 2.5). Olutiola (1976) has isolated a cellulose complex of 11 components, four high mol wt and 7 low mol wt from culture filtrates of *Penicillium citrinum*. In an enzymatic study on cellulose system of *Trichoderma viridae* two cellulases, cellulose II A and II B have been purified (Okada, 1975). To study the ability of *Thermobifida fusca* cellulases (Cel 6B, Cel 9A and Cel 5A) to bind BMCC than on Avicel, various compositions of mixtures of purified cellulases (one Endoglucanase and two CBH) were tried on the saccharification of microcrystalline cellulose (Baker et al., 1998). Two distinct cellulases (Cellulase I & II) were isolated from the water extract of a wheat bran culture of *Trichoderma koningii* (Iwasaki et al., 1964). The enzyme was precipitated by ammonium sulfate, freed of noncellulase impurities by DEAE-Sephadex and Amberlite CG-50, and fractionated on hydroxylapatite into cellulase I (eluted with 0.001M phosphate) and cellulase II (eluted with 0.1M phosphate buffer). They differ in substrate specificity (Table 2.6). *Trichoderma koningii* produces a powerful cellulase which hydrolyses native cellulose completely to glucose (Halliwell, 1965). Waksman (1991) has purified from *Sclerotinia sclerotiorum* two endoglucanases with mol wt 48KDa and 34KDa active towards CMC.

Anaerobic chytrid fungi are only found in the rumens of herbivorous animals (Orpin, 1977) and produce highly active cellulases (Borneman et al., 1989; Chen et al., 1997; Wilson & Wood, 1992; Wood et al., 1986). Ye et al., (2001) has

Table 2.4 Purification of the Endo-1, 4-ß glucanase from *Neisseria sicca SB*

Purification step	Total Protein (mg)	Total Activity (U)	Specific activity (U/mg)	Purification (Fold)	Yield (%)
Culture supernatant	40300	4040	0.100	1.00	100
Ammonium sulphate fractionation	11200	3890	0.347	3.47	96.3
DEAE-Sepharose	3190	2880	0.903	9.03	71.3
Q-Sepharose	512	293	0.572	5.72	7.25
Phenyl Toyopearl	31.4	34.3	1.09	10.9	0.85
Mono Q	9.38	17.2	1.83	18.3	0.43

Source: Moriyoshi et al., 2003

Table 2.5 Certain properties of Endoglucanases from *Neisseria sicca, Mytilus viridis, Rhodotorula glutinis* and *Volvariella volvacea*

Source of Endoglucanase	Mol wt KDa	Sub-strate	Specific activity (U/mg)	K_m (%)	V_{max} (μmol/min/ mg)	Temp. (°C)	pH	Isoelectric pH
Neisseria sicca SB (Moriyoshi et al., 2003)	41	Cellulose acetate	1.82	0.242	2.24	60	6-7	4.8
Blue mussel *Mytilus viridis* (Xu et al., 2000)	20	CMC	10.4	-	-	30-50	4-6	7.6
Rhodotorula glutinis KUJ 2731 (Oikawa, 1975)	40	CMC	-	1.1	556	50	-	8.57
Volvariella volvacea (Ding et al., 2002)	42	CMC	-	-	-	55	7.5	7.7

Table 2.6: Properties of *Trichoderma koningii* cellulases

Enzymes	Mol.wt	Inactivation by heat	Activity on		
			Glycol cellulose	Cellobiose	Filter paper
Cellulase I	26,000	60°C, 1hr	Yes	Yes	No
Cellulase II	50,000	70°C, 1hr	Yes	No	Yes

Source: Iwasaki et al., 1964

purified cellulase from ruminal fungus *Orpinomyces joyonii*, cloned it in *Escherichia coli* and purified to 88 fold by chromatography on High Q and hydroxyapatite. The polysaccharide hydrolases produced by anaerobic fungi are amongst the most active that have been described to date; they are capable of degrading a wide range of polysaccharides and can completely solubilize both amorphous and highly crystalline cellulose (Li & Heath, 1993; Selinger et al., 1996; Wubah et al., 1993). The anaerobic fungus *Piromyces equi* was isolated from the caecum of a pony (Munn, 1994; Orpin, 1981). The cellulose-hemicellulose degrading system of *P. equi* consists of a large multienzyme complex, which accounts for upto 90% of the cellulase, mannanase and xylanase activities produced by the fungus. This complex consists of atleast 10 polypeptides ranging from 50 to 190 KDa, including a 97 KDa putative scaffolding protein (Ali et al., 1995; Fanutti et al., 1995; Hazle wood & Gilbert, 1998). A new thermostable endoglucanase, inactivated only by 20% at 64°C for 6 hours in the absence of substrate was reported to be formed when the cultivation temperature of a thermophilic culture of *Allesheria terrestris* was increased from 40 to 48-49°C (Kvesitadze et al., 1996). Its molecular weight and isoelectric point were 69KDa and 6.4 respectively. The cellulase of *Aspergillus terreus* showed no heterogeneity on Sephadex G-100 or an ion-exchange chromatography; the purified enzyme hydrolyzed carboxymethylcellulose and alpha-cellulose (Pal & Ghosh, 1965). Highly cellulolytic anaerobic hyperthermophiles are found in the genera *Thermotoga* (Liebl, 2001) and *Caldicellulosiruptor* (Rainey et al., 1994)

and cellulases isolated form these organisms are often highly thermostable (Bok et al., 1998).

It has been reported that yeast, *Rhodotorula glutinis* KUJ 2731, isolated from soil effectively produce an extracellular endoglucanase. The molecular mass was found to be 40 KDa, isoelectric point 8.57, and activation energy 20.9KJ/mol. The enzyme was found to be active at temperatures 0-70°C with highest initial velocity at 50°C. The enzyme catalyzed hydrolysis of CMC with an apparent K_m of 1.1% and V_{max} of 556 µmol/ min/ mg (Table 2.5). The enzyme was capable of catalyzing the transglycosylation of p-nitrophenyl-β-cellotrioside to cellotetraose (Oikawa, 1998). Biotinylation of intact *Saccharomyces cerevisiae* cells with a non permeant reagent (Sulfo-NHS-LC-Biotin) allowed identification of seven cell wall protein that were released from intact cells by dithiothreitol (DTT). Three of these proteins were identified to be β-exoglucanase I, β- endoglucanase and chitinase (Cappellaro et al., 1998). Endoglucanases/ cellulases have been used in yeast cell wall lysis. In a study to determine the cell wall composition of *S. cerevisiae* it was found that a combination of chitinase and recombinant β-1,3-glucanase released all the chitin and 60-65% of β-1,3-glucan, recombinant endo-β-1,6-glucanase of *T. harzianum* release all the β-1,6-glucan and finally laminariase and β-glucosidase released the remaining β-1,3-glucans present in the cell wall of Saccharomyces cerevisiae (Magnelli et al., 2002).

An endo-β-1,4-endoglucanse from edible mushroom, *Volvariella volvaceae,* has been purified, characterized and expressed in *Pichia pastoris* (Ding et al., 2002).

Almin and Eriksson (1968) have developed the theoretical and semiemprical basis for a viscometric method of determining the activity of enzymes acting, preferably at random, in the degradation of polymers. This method measures the activity in absolute terms, i.e., the number of bonds broken per unit time. By this method, the molecular activity of a purified sample of *Penicillium notatum* endoglucanase, of molecular weight 78000, acting on sodium carboxymethylcellulose (DS 0.83) at 25°C has been determined to be 29 bonds per second.

Some metals inactivate endoglucanase whereas few others activate. Endoglucanase from hyphomycete *Chalara paradoxa* was inhibited by detergents, EDTA, Hg^{2+}, Ag^{2+}, some extent by 10mM Zn^{2+}, Fe^{2+} and Mg^{2+}, but stimulated by Mn^{2+} (Lucas et al., 2001). Cellulase of *Trichoderma viridae* was found to be completely inhibited by 1mM Hg^{2+}, partially by 1mM Ag^{+} and Cu^{2+}. Mg^{2+}, Fe^{2+} and several other metal ions showed no inhibition at this concentration (Okada, 1975). Many unspecific protein-denaturing inhibitors are active also against cellulases. Inhibitors, which act on free SH-groups, also inhibit cellulases. Mandels and Reese (1964) have dealt extensively with the very interesting natural inhibitors of cellulase, chiefly belonging to phenolics, tannins, and types of polymeric leucoanthocyanins. They are probably widespread in plant tissues. About one-fifth of the plants screened contained reasonable amounts more than 12 times the amount found in wood of *Eucalyptus rostrata*, the first localized source of natural cellulase inhibitors. Cellulases from different sources vary markedly in their resistance to these inhibitors. Mandels

and Reese discuss the significance of these natural inhibitors as a factor involved in the resistance of plants. Inhibitors of cellulases have possible applications as agents for protecting cellulosic materials from microbial attack (Jurášek et al., 1967).

Cellulolytic enzymes are generally considered to be formed only in the presence of cellulose. According to Mandels and Reese (1960), however not cellulose itself but the soluble cellobiose, an hydrolysis product, is the true inducer of cellulase in cellulose cultures. Other compounds having β-glycosidic linkages, such as lactose and salicin, can serve as inducers. However, not all soluble compounds with the β-glycosidic linkages are inducers. Glucose and cellobiose offered in amounts as large as those used in standard growth experiments generally depress the yield of cellulolytic enzymes (Norkrans, 1967). Horton and Keen (1966) studied the sugar repression on cellulase synthesis in the case of *Pyrenochaeta terrestris*, a fungus involved in the formation of onion pink root. *P. terrestris* has been observed to produce carboxymethylcellulase in infected onion roots. The enzyme is also produced in quantities in cultures of the fungus containing cellulose. Cellulase production would be depressed as long as glucose or other soluble carbohydrates attacked by 'constitutive' enzymes are present. The cell wall, however, constitutes a cellulose substrate, which will induce an increase in cellulase production when the plant, for some reason, no longer forms a surplus of soluble carbohydrates (Norkrans, 1967).

Cellulase has been immobilized using polyurethane foam (Chakrabarti & Storey, 1988). The enzyme exhibiting cellulolytic (endoglucanase) activity at alkaline pH

isolated form the acremonium species has been patented both for its application in improving pulp drainage and for deinking recycled paper (Anderson, 2000). The degradation of cellulose is an increasingly important problem in waste treatment. Purified endoglucanases from *Trichoderma reesei, Penicillium verruculosum* and *Chrysosporium lucknowense* ability to bind indigo has demonstrated the best washing performance in the process of enzymatic denim treatment (Gusakov et al., 2001).

Cellulases, β-glucanases and β-glucosidases of fungi are used in the production of syrups and coffee whiteners. The United Kingdom Ministry of Agricultural Fisheries and Food (MAFF) in 1982 have recommended cellulase of *Trichoderma viridae* in the permitted list, to be used in food (Robinson, 1987). Endoglucanase finds application in textile industry (Hirvonen & Papageorgiou, 2003). Disintegration of plant tissue has been experimentally provoked with cellulase preparations. Gross (1990) succeeded in preparing protoplasts by removal of cell walls from carragenophyte *Gigartina corymbifera* using cell wall digesting enzymes. Protoplasts obtained showed vigorous cytoplasmic streaming, and formed a good experimental material for attacking a number of plant physiological problems such as wall formation, ion transport, and water balance. They also serve as a useful tool in crop improvement. Cellulases are used as cell wall disintergrators in order to increase the digestibility of proteins, fruit juices, essential oils, agar-agar from seaweeds, etc. For a long time, cellulases have found application in the pharmaceutical industry as a digestive aid (Norkrans, 1967).

The baking quality of rye flour and the shelf life of rye bread can be improved by partial hydrolysis of the rye pentosans. Technical pentosanase preparations are mixtures of β-glycosidases (1,3- & 1,4-D-xylanases). Solubilization of plant constituents by soaking in an enzyme preparation (maceration) is a mild and sparing process. Such preparations usually contain exo- and endo- cellulases, alpha and beta mannosidases and pectolytic enzymes. Examples of the utilizations are: production of fruit and vegetable purées (mashed products), disintergration of tea leaves, or production of dehydrated mashed potatoes. Some of these enzymes are used to prevent mechanical damage to cell walls during mashing and thus, to prevent excessive leaching of gelatinized starch from the cells, which would make the puree too sticky. Glycosidases (cellulases and amylases from *A. niger*) in combination with proteinases are recommended for removal of shells from shrimp. The shells are loosened and then washed off in a stream of water (Belitz & Grosch, 1999). Glycanases secreted by *Aspergillus terreus* CCMI 498 and *Trichoderma viridae* CCMI 8 were effective in deinking mixed office waste paper (Marques et al., 2003). *Aspergillus niger, Aspergillus oryzae, Rhizopus delemar, Rhizopus oryzae* and *Penicillium emersonii* are the fungi capable of producing endoglucanase finding wide application in the food processing (Belitz & Grosch, 1999).

2.5 Endoglucanase from bacteria

Cellulolytic bacteria can be observed to comprise of several diverse physiological groups. (1) Fermentative anaerobes, typically gram positive (*Clostridium,*

Ruminococcus and *Caldicellulosiruptor*) but containing a few Gram negative species, most of which are not (*Fibrobacter*) (2) Aerobic Gram postitive bacteria (*Cellomonas* and *Thermobifida*) and (3) Aerobic gliding bacteria (*Cytophaga* and *Sporocytophaga*). Generally only a few species within each of the above named genera are actively cellulolytic. The cellulolytic capability among the organisms that differ in oxygen relationship, temperature and salt tolerance indicates wide availability of cellulose across natural habitats. In aquatic habitats, hydrolysis of cellulose by secreted cellulases may be feasible e.g. by the predominance of *Cytophaga*, which is known to actively secrete cellulases in culture (Kauri & Kushner, 1985; Li & Gao, 1997; Malek et al., 1988). Two alkaline carboxymethylcellulases were partially purified and exhibited pH optima of 10.0 and molecular mass of 54 and 46KDa. The enzymes were stable upto 60 and 80°C, respectively (Garnier-Sillam et al., 1985). Horikoshi's group has characterized another cellulase from alkalophilic *Bacillus* strain 1139. It exhibited a pH optimum of 9.0 and still showed much activity at pH 10.5 (Bracke et al., 1978). This extracellular enzyme had a particularly high content of aspartic acid. Cloned into *Escherichia coli* the enzyme had the same properties as the cellulase from *Bacillus* strain 1139, but its molecular mass was 94 rather than 92 KDa, perhaps indicating processing at a different sites from that in the *Bacillus* strain of origin (Bracke & Markovetz, 1978). The evidence that non-cellulolytic bacteria *Clostridium acetobutylicum* contains a complete cellulosomal gene cluster system that is not expressed, due to disabled promoter sequences (Schwarz, 2001), complicate the taxonomic picture. Among the bacteria, there is a distinct

difference in cellulolytic strategy between the aerobic and anaerobic groups. With relatively few exceptions (Rainey et al., 1994; Svetlichnyi et al., 1990) anaerobes degrade cellulose primarily via complexed polycellulosome organelles of the thermophilic bacterium *Clostridium thermocellum* (Schwarz, 2001). A strictly anaerobic Gram negative bacterium *Pervotella spp.* is found to play a significant and essential role in the utilization of plant cell wall materials. A broad specificity endoglucanase/ xylanase were partially purified along with a 66KDa endoxylanase by a two step HPLC procedure. The non-concentrated periplasmic fraction from *Pervotella bryantii* cultivation medium was separated into six fractions in a gradient HPLC system employing anion exchange principle. In the second step, fraction four was further separated into four fractions (A, B, C, D) by HPLC and these were identified as enzymes of molecular weights 88, 66, 45 and 29 KDa by SDS PAGE. One fraction (4B) was identified as endoxylanase (66KDa) and the next (with two bands) as a broad specificity endoglucanase/ xylanase (Logar et al., 2000).

Wharton's et al., (1965) investigated celllulolytic activity in cockroach, *Periplaneta americana* and demonstrated cellulases of both insect origin and bacterial origin in the alimentary tract. The former originated in the salivary glands and the latter was concentrated in the midgut. CMC was the substrate. No attempts were made to isolate cellulolytic bacteia or to estimate the number present. Isolates were identified as *Klebsiella oxytoca, Clostridium freundii,* and species of *Eubacterium, Clostridium*, and *Serratia*. None of the isolates, nor a cellulolytic reference strain of *Ruminococcus albus* used as a control, digested Whatman

No.1 filter paper in these experiments. When enzyme assays were performed with ball-milled cellulose as substrate using supernatant fluids from cultures grown with CMC, the cellulolytic activity of all strains but one was 30-75% less than when CMC was the substrate (the activity of *Clostridium* remained about the same). Although these bacteria were grown with CMC and the presence of a C_x-cellulase would be expected, the activity seen with ball milled cellulose indicates that C_1-cellulase activity may also have been present. Alternatively, it could indicate that the ball-milled cellulose also contained polymeric degradation by the C_x- enzyme. Bignell (1977) showed that [^{14}C] cellulose is degraded in the hindgut of *Periplaneta americana*. Feeding of antibiotics sharply reduced the $^{14}CO_2$ evolution. This indicates that the hindgut bacteria had a role in degradation of this polysaccharide. Breznak (1982, 1984) found no convincing evidence implicating bacteria in cellulose degradation in the higher termites that possess cellulolytic protozoa.

A 100KDa protein with endoglucanase activity was purified from Triton X-100 extract of cells of the thermoacidophilic Gram-positive bacterium *Alicyclobacillus acidocaldarius*. The pH and temperature optimum was found to be 4 and 80°C respectively (Eckert & Schneider, 2003). The enzyme has been sequenced to the gene level. *Bacteroides succinogenes*, because of its high cell numbers and ability to ferment cellulose rapidly, is one of the more important cellulolytic organisms in the rumen. It is the only cellulolytic organism able to actively digest undegraded cotton fiber (Halliwell & Bryant, 1963). The cellulase of these organisms appears to be associated with the outer surface of the cell in that the

cellulose being degraded must be in close proximity to the bacterial cells (Macy & Probst, 1979). Carboxymethylcellulase activity of this bacterium was studied. The partially purified enzyme gave an optimum temperature of 50°C and optimum pH of 5.6-6.6.

Aerobic cellulose degraders both bacteria and fungi, utilize cellulose by producing extracellular cellulase enzymes that are freely recoverable from culture supernatants (Rapp & Beerman, 1991; Schwarz, 2001). They are occasionally present in complexes at the cell surface (Bond & Stutzenberger, 1989; Wachinger et al., 1989).

Four electrophoretically distinct fractions from *Cellvibrio gilvus* cleaved cellohexose, cellohexitol, and cellopentitol at the second and third linkage from the non-reducing end (Cole & King, 1964). Electrophoresis in a block of starch grains separated five components with activity toward carboxymethylcellulose from an enzyme preparation from *Streptomyces gilvus*; according to immuno diffusion tests, three of them were immunologically identical (Enger & Sleeper, 1965).

An alkaline cellulase produced by alkalophilic *Bacillus spp. N6-27* was purified by ammonium sulphate fractionation, sepharose CL-4B hydrophobic interaction chromatography and Bio-gel P-150 chromatography (Tian & Wang, 1998). Its mol wt on SDS PAGE was 94 KDa and pI by PAGE - IEF was 4.2. The optimum temperature and pH for the enzyme on CMC was found to be 55°C and 8.5 respectively. The enzyme activity was stable under 50°C and in the pH ranges

6-11. Fe^{2+}, Cu^{2+} and Hg^{2+} were strongly inhibited by this enzyme. An endoglucanase was reported to be purified from *Clostridium thermocellum* by (Ng & Zeikus, 1981). The enzyme purified to 22 fold had isoelectric point, optimum temperature and optimum pH of 6.72, 62°C and 5.2 respectively. The enzyme lacked cysteine and was low in sulphur containing aminoacids. The enzyme had increased activity towards cello-oligosaccharides with increasing degree of polymerization. Several extra-cellular hydrolytic enzymes (xylanase, endoglucanase, β-xylosidase, β-glucosidase) were produced by a *Clostridium* strain PXYL1 isolated from cold- adapted cattle manure digester at 15°C (Akila & Chandra, 2003). An extracellular cellulase produced by *Bacillus brevis*, isolated from the soil was purified by ultrafiltration and Sephadex G-200 column chromatography. The production increased almost five times on addition of galactose in culture medium and was optimum at pH 5.5 and 37°C and 175 rpm speed using environmental orbital shaker (Singh & Kumar, 1998). The location of cellulase in *Cellvibrio fulvus* was studied by Berg (1975). It is reported that the enzyme was repressed by glucose and formed at a constant differential rate on cellobiose and amylose. An endoglucanase was purified from cellulolytic thermophilic anaerobic bacteria by Creuzet and Frixon, (1983). The optimal pH was 6.4 and the enzyme was isoelectric at pH 3.85. It was found highly thermostable; it retained 50% of its activity after 1 hour at 85°C. Hydrolysis of CMC was detected by a rapid decrease in viscosity but slow liberation of reducing sugars, indicating endo-enzyme activity. A 100KDa protein with endoglucanase activity was purified from Triton X-100 extract of cells of

thermoacidophilic gram-positive bacteria *Alicyclobacillus acidocaldarius* (Eckert & Schneider, 2003). The enzyme showed activity towards CMC with pH and temperature optima of 4 and 80°C respectively.

"Fungi - like" prokaryotes such as Actinomycetes and the related Corynebacteria (*Cellulomonas*) might degrade cellulose, according to a mechanism similar to that of fungi, with cellulolytic enzymes that can be found non-associated in the culture medium (Coughlan & Ljungdahl, 1988). Conversely, in many anaerobic bacteria (rumen bacteria, *Clostridium thermocellum*), the various components are found in tightly associated multimolecular complexes, whose quarternary structure seems to be a key feature responsible for the efficient degradation of crystalline cellulose (Lamed & Bayer, 1988). The cellulase complex of *Clostridium thermocellum* is found to contain at least 14-18 different polypeptides forming a very stable extracellular structure termed cellulosome (Coughlan et al., 1985; Lamed et al., 1983a,b). In a study on cellulose digestion of roe deer (*Capreolus capreolus*) it was found that cellulase activities were lowest in winter when the cellulose concentration in rumen was the highest (Deutsch, 1998).

The best-studied species of cellulolytic aerobic bacteria belong to the genera *Cellulomonas* and *Thermobifida* (formerly *Thermomonospora*). *Cellomonas* species are coryneform bacteria that produce at least six endoglucanases and at least one exoglucanase (Chaudhary et al., 1997). The thermophilic filamentous bacterium *Thermobifida fusca* (formerly *Thermonospora fusca*) is a major cellulose degrader in soil. Six cellulases, three endoglucanses (E1, E2 & E5),

two exoglucanases (E3 & E6) and an unusual cellulase with both exo and endoglucanase activity (E4) have been isolated. The latter enzyme has high activity on BMCC and also exhibits synergism with both the other *Thermomonospora fusca* endoglucanases and exoglucanases (Irwin et al., 1993). The thermophilic and hyperthermophilic prokaryotes represent a unique group of microorganisms that grows at temperatures that may exceed 100°C. Several cellulolytic hyperthermophiles have been isolated during the past decade (Bergquist et al., 1999). However, no cellulolytic thermophilic archaea have been described, although archaea that can grow on cellobiose and degrade other abundant polysaccharides such as starch, chitin and xylan, have been isolated (Driskill et al., 1999; Sunna et al., 1997). Only two aerobic thermophilic bacteria have been described that produce cellulases: *Acidothermus cellulolyticus* (an actinomycete) and *Rhodothermus* (Sakon et al., 1996). A thermostable alkaline cellulase activity was detected in a culture medium of a strictly alkaliphilic *Bacillus (KSM- S 237)* by Hakamada et al., (1997). The alkaline enzyme of optimum pH 8.6-9.0 and optimum temperature 45°C was stable upto 50°C and more than 30% of the original activity was detectable after heating at 100°C.

Endoglucanase and protease of *Oerskovia xanthineolytica* act together in the yeast cell lysis (Scott & Schekman, 1980). Cellulase has been used to inhibit biofilm formation by a pathogenic bacteria *Pseudomonas aeruginosa*, commonly found in Medicinal plants (Loiselle & Anderson, 2003). Microorganims produce multiple enzymes to degrade plant cell materials, known as enzyme systems (Warren, 1996). Endoglucanases of *Bacillus circulans* and *Bacillus subtilis* are

used in food processing (Belitz & Grosch, 1999). Cellulase can be used instead of pumice stones for "stone washing"- a process that is used to give blue jeans an abraded and worn in look. For this purpose Genencor International has created a cellulase, called Indi Age Neutra L Enzymes obtained from *Streptomyces lividans*. It is known to work best within a specific temperature range of 45 – 55°C and pH range of 6-8 (Chen et al., 2000).

Prior reviews consider the complexed cellulases of anaerobic bacteria (Bayer et al., 1998; Bayer et al., 1994; Doi et al., 1994, 1998; Felix & Ljungdahl, 1993; Leschine, 1995; Robson & Chambliss, 1989; Schwarz, 2001; Shoham et al., 1999) noncomplexed fungal and bacterial cellulases (Coughlan, 1990; Knowles et al., 1987; Ryu & Mandels, 1980; Stutzenberger, 1990; Teeri, 1997; Teeri et al., 1998; Wood, 1992).

2.6 Plant endoglucanase

Endoglucanases are widely distributed in plant products namely seeds and malts. However, comparatively, less work has been reported. The plant endoglucanases are capable of hydrolyzing polysaccharides found in the plant and fungal cell walls, thus functioning in plant development and in defense of the plant against fungal diseases (Simmons, 1994). Two forms of endoglucanases of molecular weight approximately 20,000 and 70,000 have been identified in growing regions of *Pisum sativum* epicotyls treated with auxin. One cellulase is buffer soluble, the other buffer insoluble but extractable with high salt concentrations. They were purified by DEAE cellulose chromatography,

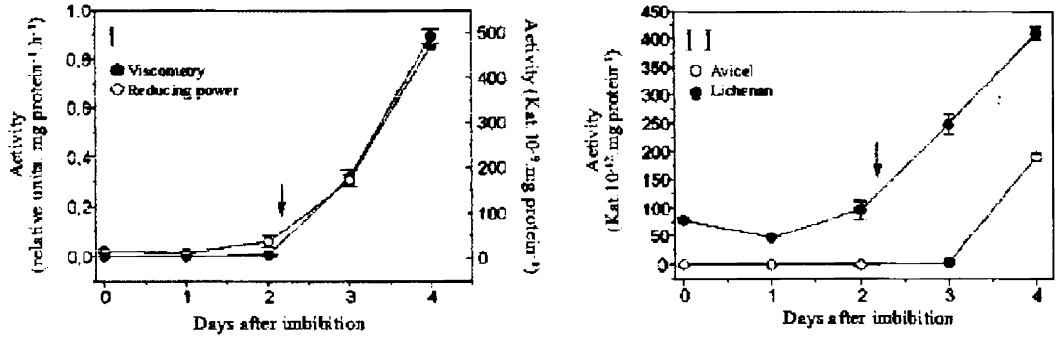
Sephadex gel filtration and ultrafiltration methods. Both the enzymes hydrolysed CMC with optimum pH 5.5 to 6.0. Aminoacid analysis revealed that one buffer soluble enzyme is relatively rich in Gly, Ala and Val and deficient in Cys, Tyr and Phe compared to the other buffer insoluble enzyme (Byrne et al., 1975). An endoglucanase was purified from ripe strawberry fruit using affinity chromatography. Its mol. wt on SDS PAGE was determined to be 54KDa. Its pH optimum and K_m against CMC was determined as 5.0-7.0 and 1.3 mg/ml respectively (Woolley, 2001).

Laminarin hydrolyzing activity (ß-1, 3-EG EC 3.2.1.39) was reported to be developed in the endosperm of tomato (*Locopersicon esculentum*) seeds following germination. The enzyme was basic with isoelectric point greater than 10 and the apparent molecular mass was estimated to be 35KDa by SDS-PAGE (Morohashi & Matsushima, 2000). Two endoglucanase genes had been isolated from *Pinus radiata* and expressed (Loopstra et al., 1998). Both proteins contained domains conserved in plant and bacterial endoglucanases. They showed strong similarity to each other and higher similarity to and endoglucanase cloned from tomato pistils. Studies have shown that a 66 KDa endo-1,4-β glucanase is present in the endosperm of *Euphorbia heterophylla L.* The carboxymethylcellulase activity decreased approximately by 66% in extracts of endosperm containing isopropanol or ethanol. The endoglucanases were isolated from endosperm extracts using ammonium sulphate fractionation followed by Sephacryl S-100-HR chromatography resulting in two peaks. Peak I was purified about 15 fold by DEAE Sephadex A50 followed by affinity

chromatography (CF-11 cellulose) and peak II purified to 10 fold by CMC chromatography (Suda & Giorgini, 2003). Activity of cell wall hydrolases in the germinating seeds of *Euphorbia heterophylla L* has been studied. The activities were very low or not detected during pre-emergence period (time interval before 2.2 days from the start of imbibition) and increased in the post emergence period (time interval after 2.2 days from the start of imbibition). Figure 2.2 (I and II) shows the endoglucanase activity on CMC and avicel/lichen, obtained by the authors.

Plant endoglucanases in glycosyl hydrolase family 17 include endo-(1,3) : (1,4) - β- glucanase (E.C.3.2.1.73) and endo-1,3-β- glucanase (E.C.3.2.1.39) (Henrissat & Bairoch, 1993). These enzymes hydrolyze polysaccharides found in the plant and fungal cell walls, thus functioning in the plant development and in defense of the plant against fungal diseases (Simmons, 1994). Maize coleoptile endoglucanase has been purified and partially sequenced (Inouhe & Nevins, 1991). Endoglucanase has been isolated from the cell walls of *Zea mays* seedlings (Hatfield & Nevins, 1986). The activity of cell wall hydrolases such as endo-β-1, 3-glucanases during the radicle emergence stage of germination may be related to the softening of the tissue in the micropylar region, where the protrusion of the radicle occurs (Leubner-Metzger et al., 1995; Nonogaki & Morohashi, 1996; Sánchez & de Miguel, 1997; Nonogaki et al., 2000; de Miguel et al., 2000; Leubner-Metzger & Meins Jr., 2000).

Figure 2.2 Time course of endoglucanase from *Euphorbia heterophylla* endosperm



Graph I shows the activity of endoglucanase on CMC determined by viscometry (left scale) and by reducing power production (right scale). Graph II shows the activities of endoglucanase on Avicel and lichenan. The arrows indicate average germination time.

A wall bound endo-(1,4)-beta glucanase obtained from a preparation of the cell walls of suspension cultured poplar cells was purified to electrophoretic homogeneity by cation- exchange, hydrophobic and gel filtration chromatography. The enzyme gave a molecular mass of 47 KDa by SDS PAGE and 48 KDa by gel filtration on Superdex 200 pg with isoelectric point 5.6, optimal pH 6.5, K_m 1.2 mg/ml and V_{max} 280 units (Ohmiya et al., 1995).

Cellulase is abundantly present in the pulp of ripe bananas (Peumans et al., 2000). This 1, 3- glucanase showed similarity with other plant β-glucanases with respect to aminoacid sequence, structure and biological activity. Fabin, a novel calcyon like and glucanase like protein with mitogenic, anti-fungal and translation inhibitory activity was isolated from broad beans *Vicia faba* and properties studied (Ng & Ye, 2003). Cellulase activity is found to be increased during ethylene induced abscission of leaves and ripening of fruits in pepper plants (Ferrarese et al., 1995). Endo-β-1,4-glucanase and β-galactosidase are important enzymes associated with the mobilization of xyloglucans (Edwards et al., 1985; Crombie et al., 1998; Tiné et al., 2000). Few plant cellulases are capable of degrading crystalline cellulose (Rose & Bennet, 1999). Cellulase of *Phaseolus vulgaris* has been studied by Durbin and Lewis (1988).

Cellulase is thought to be maximally active in vivo during spore germination (Cotter et al., 1969). During the swelling stage of germination extracellular cellulase having a high specific activity is first detected. The time of appearance

of this extracellular enzyme coincides with that of the dissolution of both the outermost mucopolysaccharide layer and the two middle cellulosic wall layer (Cotter et al., 1969; Hemmes et al., 1972). Cellulase activity in vitro during differentiation parallels the accumulation of its substrate in vivo (Rosness, 1968). However, it is undetectable in extracts prepared prior to sorocarp stage of development (Killick & Wright, 1974).

In the brewing process, β-glucans from barley increase wort viscosity and impede filtration. Enzymatic endo-hydrolysis reduces viscosity. Exocellobio-hydrolase of *Trichoderma reesei* has been expressed and biochemical characteristics studied in transgenic tobacco (Dai et al., 1997; Dai et al., 1999).

2.7 Endoglucanase isolated from insect gut

Cellulases have been isolated from the gut of several insects. Endoglucanases are synthesized in the esophageal glands of the cyst nematodes *Globodera rostochiensis* and *Heterodera glycines* (Smant et al., 1998). Cyst nematodes are obligatory plant parasites and the identified endoglucanases facilitate intracellular migration through plant roots by partial cell wall degradation. It has been reported that termites can exist for long periods on a diet of pure cellulose when the normal flora are present (Breznak & Pankratz, 1977). No cellulolytic bacteria were found in the larvae of *Costelytra zealandica*, the grass grub beetle a root feeder on pasture plants (Bauchop & Clarke, 1975). Cellulase of both insect and bacterial origin was identified in the alimentary tract of *Perplaneta americana* (Wharton et al., 1965). An endoglucanase with specific activity 150 μmol/ min/ mg protein against CMC and molecular weight 47 KDa have been isolated from

the gut of larvae of yellow spotted longicorn beetle, *Psacothea hilaris*. The optimal pH of this cellulase was 5.5, close to the pH in the midgut of *P. hilaris* larvae (Sugimura et al., 2003). The deduced aminoacid sequence of *P. hilaris* showed high homology to the members of glycosyl hydrolase family 5 subfamily 2. Watanabe et al., (2002) have purified an endoglucanase from the hindgut of an Australian mound-building termite, *Coptotermes lacteus*. The hindgut extract had a peak separate from those for extracts obtained from the salivary glands and the midgut based on Sephacryl S-200 gel chromatography demonstrated an origin different from the endogenous endoglucanases of the termite itself. It showed high homology to endoglucanases from glycoside hydrolase family 7. A multienzyme distribution of endoglucanase activity was found in the digestive system of a worker caste of the lower termited *Coptotermes formosanus* by zymogram analysis. Distribution analysis showed that 80% of its activity was localized in salivary glands from where only one component (Endoglucanase-E) was secreted into the digestive tract. Its molecular mass, optimal pH, temperature, isoelectric point and K_m were reported as 48KDa, 6.0, 50°C, 4.2 and 3.8 (mg/ml on CMC) respectively (Nakashima and Azuma, 2000). The N-terminal aminoacid sequence of Endoglucanase-E showed similarity with fungal endoglucanase of glycosyl hydrolase family 7 rather than the other insect Endo-β-1,4 - glucanases of family 9.

2.8 Cellulases of mollusks

The first unequivocal action of cellulase observed was that the hepatopancreas of Weinberg snail, *Helix pomatia* exhibited a slow action on cotton cellulose and

a much more rapid reaction on regenerated celluloses. Studies on snail cellulose have been carried out extensively by (Pigman, 1951). Table 2.7 shows the works done on cellulases from mollusks. Of the thirty of more enzymes associated with the digestive tract of *Helix* (Holden & Tracey, 1950; Myers & North-cote, 1958), more than twenty are carbohydrases which are reported to include cellulases (Myers & Northcote, 1958) as well as a variety of glycosides. A wide range of carbohydrases has also been demonstrated in the snail, *Tegula funebralis* and it has been suggested that *Tegula* owes its abundance in the upper littoral zone in part to this wide array of enzymes which enables it to digest a wide variety of algae (Galli & Giese, 1959). Apart from *Tegula* and *Helix*, cellulases have also been reported from many genera including *Patella*, *Littorina* and *Aplysia* (Stone & Morton, 1958), *Dolabella* (Hashimoto & Onoma, 1949), *Melanoides* (Fish, 1955), *Oncomelania* (Wrinkler & Wagner, 1959) and *Strombus* and *Pterocera* (Younge, 1932). Myres and Northcote (1959), have suggested that more than one enzyme may be involved in the digestion of cellulose in the gut of *Helix* and that, although one or more of these may be produced by the gut flora, it is possible that others are produced by the animal. (This view is based on the properties of three fractions obtained during an attempt to purify the cellulase of *Helix pomatia*. All three fractions possessed the same order of cellulase activity, but their proteins showed different chromatographic mobilities, suggesting that at least three cellulolytic enzymes are present). Florkin and Lozet (1949), however, have reported that extracts of the digestive diverticula or crop of Helix show no evidence of cellulolytic activity. But in contrast, Galli and

Table 2.7 Occurrence of cellulases in Molluscs.

Molluscs	Reference
Snail (*Helix pomatia*)	Pigman (1951)
Giant snail (*Achatina fulica*)	Maeda et al., (1996)
Snail (*Levantina hierosolyma*)	Parnas (1961)
Shipworm (*Bankia indica*)	Nair (1955)
Clam (style)	Lavine (1946)
Blue mussel	Xu et al (2000)

Giese (1959) have concluded that the cellulolytic bacteria present in the gut of *Tegula* are relatively unimportant. Only four strains of the bacteria isolated from the gut of this animal were capable of breaking down structural carbohydrates more readily than extracts of the digestive diverticula and crop, and these were present in small numbers only. The extracts contained a certain amount of bacterial protoplasm along with the animal tissue, but tests on the main residual bacterial species present showed it to be incapable of attacking any of the algae or algal polysaccharides examined.

Parnas (1961) has investigated the source of cellulases in the snail *Levantina hierosolyma* by comparing the cellulolytic activity of normal snails with that of snails whose digestive tract had been sterililzed with antibiotics. This was achieved by feeding the snails on a mixture of sterile flour and antibiotics and transferring them every 24 hours to fresh petri dishes. Only when culture test showed that the feces were sterile were extracts made of the salivary glands, but in snails treated with antibiotics the activity persisted only in the digestive diverticula. From this diverticula of *Levantina* is produced by the animal whereas the cellulolytic activity of the crop and salivary glands may result either from bacteria or from the enzymes from the digestive diverticula. Thus, there now seems to be good evidence that at least some gastropods possess the ability to secrete a cellulase.

About 50 ml of contents were isolated from the guts of Weinberg snails. When the 50ml was dried over calcium chloride, about 7.5g of dry powder was

obtained. Cellulase splitting enzymes of *Helix pomatia* was isolated in several steps: grinding the intestinal pieces with toluene and sand, filtering through asbestos, dialyzing this solution for several days, then treated with 20 ml of 0.2N acetate mixture at pH 3.5 and a suspension of aluminium metahydroxide (corresponding to 370mg of alumina) and water added to make a volume of 100ml. The residual solution was then treated with 10ml of alumina suspension (corresponding to 250mg of alumina) and centrifuged again. Residual solution contains high cellulase content (de Stevens, 1955). Cellulase has been purified and characterized from giant snail *Achatina fulica* by Maeda et al., (1996). Endogenous cellulases have been isolated from marine mollusc, *Littorina brevicula* (Purchon, 1977; Kiesov, 1982). The Antartic krill has a very active system of digestive enzymes, capable of hydrolyzing a large variety of polysaccharidases. The activity of these enzymes is positively correlated with the feeding intensity of the crustacean and activities are comparatively high at low temperatures (Kolakowski & Sikorsi, 2000). High activity of carboxymethylcellulase was found in Antartic krill, *Euphausia superba* (Chen & Gau, 1981). A 20 KDa endoglucanase of whole blue mussel was purified to homogeneity by a combination of acid precipitation, heat precipitation, immobilized metal ion affinity chromatography, size exclusion chromatography and ion exchange chromatography (Xu et al., 2000). This endoglucanase was identified as a single polypeptide chain with 181 aminoacids cross-linked with six disulfide bridges. Its isoelectric point as estimated by isoelectric focusing in a PAGE was 7.6. The pH optimum curve of blue mussel endoglucanase is shown

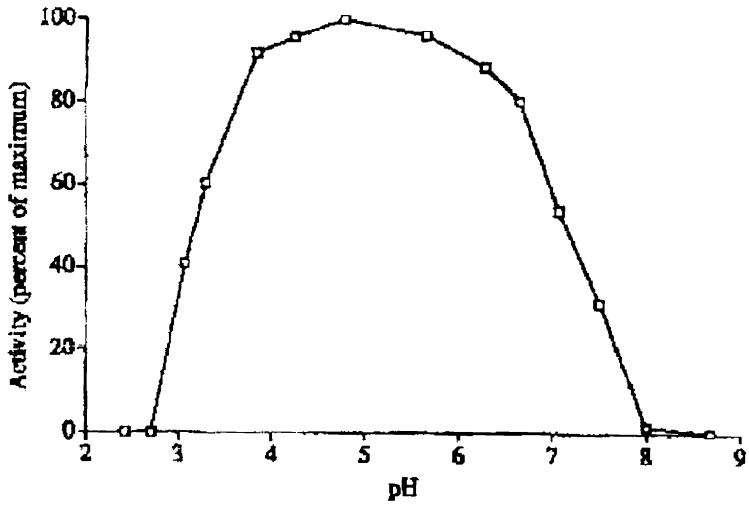
in Figure 2.3. The enzyme could withstand 10 minutes at 100°C without irreversible loss of enzymatic activity. Aminoacid sequence based classification groups it under glycoside hydrolase family 45, subfamily 2. The characteristics of the endoglucanase are shown in Table2.5.

2.9 Cellulase digestion in mollusks

The crystalline style present in the digestive system of most bivalves is largely composed of mucoproteins, carbohydrates, inorganic compounds and water. More than 90% of the dry matter of the style is protein and carbohydrate (Bailey & Worboys, 1960; Kristensen, 1972; Shahul-Hameed, 1986). Irrespective of the species, the ratio between protein and carbohydrate is approximately 3:1 (Yellowlees, 1980). The proteins in the crystalline style are crystalline. Inside the stomach the crystalline style softens and dissolves slowly, releasing a variety of digestive enzymes into the stomach. The stomach wall and the other digestive glands may also release enzymes into the stomach. Crystalline style breaks down spontaneously under a variety of physico-chemical conditions. At the same time the dissolution of the styles acidifies and lowers the viscosity of the mucoid contents of the stomach. In all bivalves, the style is continually being used up and renewed (Kristensen, 1972). The enzymes of the style are secreted by the epithelium of the sac and the style can be reabsorbed when it is not needed. It is often absent in animals that have not fed recently.

The crystalline style revolves in the stomach to perform functions like mixing and food digestion and in the process it dissolves liberating several enzymes into the

Figure 2.3: Graph showing the pH optimum for the blue mussel Endoglucanase (Xu et al., 2000)



stomach. These enzymes initiate a preliminary phase of extracellular digestion in the stomach and are capable of liberating reducing sugars from phytoplankton as well as from natural particulate detritus (Lucas & Newell, 1984). The carbohydrases of the crystalline style not only catalyze extracellular digestion in the stomach, but also participate in intracellular digestion in the digestive diverticula (Brock & Kennedy, 1992). When the particles are broken down sufficiently, they are carried on ciliary tracts in the stomach to the digestive diverticulum for intracellular digestion. Digestive cells in the diverticulum take up small food particles together with digestive enzymes into food vacuoles within the cells where nutrients can be used directly. The undigested particles and waste products are carried and stored by the intestine prior to evacuation from the anus through the exhalant siphon.

Thus the review describes briefly the work carried out in cellulases and its applications. The fact that endoglucanases have been identified in molluscs and that no work has been reported in green mussel endoglucanase, show that study of this aspect is an interesting topic of research. The thesis describes a simple procedure for purification, isolation and study of characteristic properties of the enzyme endoglucanase from green mussel, *Perna viridis*.

3. MATERIALS

3.1 Green mussel

Initially a trial was conducted to evaluate the endoglucanase activity in *Perna indica* and *Perna viridis*. Based on the result, *Perna viridis* was used throughout the experiment as the source of endoglucanase. Mussel was bought from Tikkodi of Calicut region in live condition. The mussel was cut open and the digestive gland excised and kept in chilled condition (0-4°C). On an average, large sized mussel (approximately 75g meat weight) gave about 1-2 g of digestive gland, giving a yield of 1.3 - 2.6% gland tissue. About 50g of gland were used in each batch for enzyme isolation.

3.2 Glasswares

All glasswares were washed first with water and detergent and further cleaned by soaking in 5% nitric acid for 24 hours and finally rinsed with deionised water (Milli Q water system [3.3.2.]).

3.3 Reagents and chemicals

All chemicals used were of the highest purity. The column chromato-graphic materials Sephadex G-25, and Sephadex G-100 were from Pharmacia Fine Chemicals, Uppasala, Sweden. The substrate carboxymethylcellulose, Tris-HCl, SDS, EDTA, protein standard (Bovine serum albumin) was from E-Merck India Limited, Mumbai.

The molecular weight protein markers, aminoacid standards, the reagents for aminoacid analysis, Tryptophan standard, Acrylogel (30%) were products of Sigma (St. Louis, MO, U.S.A.). Other reagents used for experiments were analytical grade reagents or the purest available unless otherwise stated and were purchased from Merck India limited and Sisco Research Laboratories private limited, Mumbai.

Reagent water : Deionised water from Milli-Q Reagent water system, which gave water of conductivity 18.2μS was used for the preparation of all reagents, standards and dilution water in all aspects of analysis.

3.4 Analytical equipments:

1) **Spectronic Genesys 5**: All spectrophotometric/ colorimetric estimations were done using Spectronic Genesys 5, Spectronic Instruments Inc. Analytical products division, Rochester, New York.

2) **Homogenizer**: The digestive gland was homogenized in acetate buffer using homogenizer, Janke and Kunkel Gmb H and Co KG IKA Labortechnik Staufen, Germany.

3) **Analytical balance:** All chemicals used in the analysis were weighed accurately using analytical balance, Sartorius, Germany.

4) **Chromatographic columns:** Sephadex G100 and G25 chromatography was performed with columns 45 cm x 1.7 cm and 30 cm x 3.1cm respectively. The columns were from Pharmacia Fine Chemicals, Uppasala, Sweden.

5) **High speed refrigerated centrifuge (Model No C24 and Model No C8C):** Separation of carboxymethylcellulose after assay and all other centrifugations (at 4°C) were performed with C8C centrifuge and refrigerated centrifuge Model No C24 respectively. The centrifuges were from REMI Instruments, Mumbai.

6) **Gel electrophoresis apparatus:** Electrophoresis was performed on polyacrylamide using vertical gel electrophoretic apparatus, BIO RAD Laboratories, USA.

7) **Water bath:** Water bath of heating equipments manufacturing company, Chennai was used for color development of assay mixture and for denaturation.

8) **Air oven:** Hydrolysis of endoglucanase to aminoacids was done using air oven Labline, Mumbai

9) **Incubator:** The assay mixture was incubated at the required temperatures using incubator purchased from Beston Industries, Cochin.

10) **Lyophilizer:** The immobilized endoglucanase was lyophilised using lyophilizer, Model No. TSI51, Hightech Instruments & System, Bapuji Nagar, Trivandrum.

11) **Digital pH meter:** The pH was read in a digital pH meter, Cyberscan 500 purchased from Selby Biolabs, Australia.

12) **Aminoacid analyzer:** Aminoacid composition of endoglucanase was determined using Shimadzu liquid chromatograph LC-10AS with FLD 6A

fluorescence detector (Shimadzu corporation, Japan), Spinco Laboratory Private Limited, Chennai.

13) **Vortex genie:** For immobilisation of endoglucanase in chitosan mixing was done in a vortex genie, Scientific Industries, INC, Bohemia, NY, USA.

14) **Manual photometer SQ 118:** For determination of isoelectric point, turbidity of the solution was measured using Manual photometer SQ 118, purchased from E. Merck, Frankfurter Straße, Darmstadt, Germany.

15) **Milli Q:** The deionised water used in all aspects of analysis was obtained by passing deionised water through a Milli Q reagent water system, Millipore India Ltd, Peenya, Bangalore.

4. METHODS

4.1 Preparation of crude enzymes

About 50 g of the digestive gland of green mussel was homogenized in 100 ml of 0.1 M sodium acetate buffer [4.2.3.2] for 15 minutes. The contents were centrifuged at 6000 g for 15 minutes in a refrigerated centrifuge. The supernatant was used as crude for isolation of endoglucanase.

4.2 Assay of endoglucanase activity

Endoglucanase activity was routinely measured according to the method of Wood and Bhat (1998) after slight modification in the procedure described under 4.2.4.

4.2.1 Principle

The enzyme cleaves carboxymethylcellulose releasing glycosylic moieties which gets oxidized in an alkaline milieu by forming orange-yellow compound with 2-hydroxy-3,5-dinitrobenzoic acid (DNSA). These orange-yellow compounds were measured at a wavelength of 530nm and the released reducing ends were quantified with glucose standard curve.

4.2.2 Reaction conditions and expression of enzyme activity.

The reaction mixture was assayed in 0.1M sodium acetate buffer (pH 5) with 1% CMC by incubating at 40°C for 30 minutes.

After assay the enzyme activity is expressed in terms of units (μmoles of glucose released/ml/min). One unit of endoglucanase activity is thus defined as the amount of enzyme that released 1μmole of reducing moieties from CMC under the above reaction conditions.

Specific activity is defined as the number of μmoles of glucose released/ min /mg of protein.

4.2.3 Reagents

4.2.3.1 Carboxymethylcellulose (1%)

Carboxymethylcellulose was used as substrate for enzyme assay. The substrate was prepared by dissolving 1g of CMC in 50 ml acetate buffer pH 5.0 by heating in a water bath for 10 min under constant stirring. After cooling to room temperature, the solution was made upto 100ml with pH 5.0 acetate buffer. The substrate could be stored at room temperature for maximum life of 4 weeks.

4.2.3.2 Acetate buffer 0.1M, pH 5.0 with 0.04% Tween 20.

0.1M acetate buffer was prepared by dissolving 8.203g of anhydrous sodium acetate and 2.94g (200mM) of $CaCl_2.2H_2O$ in approximately 900ml of deionised water and the pH of the buffer was adjusted to 5.00 $\pm$ 0.02 with 25% hydrochloric acid. 4ml of 10% Tween 20 (4.2.3.7) was added and the solution made upto 1000ml with deionised water. The buffer is stable and can be stored at room temperature for two weeks.

4.2.3.3 Sodium/ Potassium hydroxide

16g of sodium hydroxide and 22.4g of potassium hydroxide was dissolved in deionised water and made upto 100ml with the same. This could be stored in a plastic bottle for indefinite time.

4.2.3.4 DNSA reagent

About 2.5 g of DNSA was dissolved in 150ml of deionised water. To this 25ml Sodium/ potassium hydroxide (4.2.3.3) was added drop-wise. Then 75g of potassium sodium tartarate tetrahydrate (Rochelle's salt) was added and the solution cooled to room temperature and filled upto 500ml with deionised water. The reagent was stored at room temperature in dark. Maximum storage life is one month.

4.2.3.5 100mM Glucose stock standard solution

1.8016 g of D glucose was dissolved and made upto 100ml with acetate buffer (4.1.3.2). Molecular weight of glucose: 180.16.

4.2.3.6 Working standard solution:

Glucose standard stock solution (4.2.3.5) was diluted to give concentrations of 5, 10, 15, 20 and 30 micromole per ml of the solution.

4.2.3.7 10% Tween 20:

10 g of Tween 20 was made upto 100ml with deionised water. Stored at room temperature. Maximum storage life is one week.

4.2.4 Procedure

50 µl of the enzyme solution was added to 0.5ml of 1% CMC dissolved in 0.1 M sodium acetate buffer, pH 5.5. After incubation at 40°C for 30 min, 1ml of DNSA reagent was added, kept for 5 minutes in a boiling water bath and cooled on ice for 5 minutes. The contents were centrifuged at 3000 rpm for 10 minutes to remove unreacted carboxymethylcellulose and diluted with 1.5ml of deioised water. The degree of enzymatic hydrolysis of the CMC was determined spectrophotometrically by measuring the absorbance at 530nm. A reaction blank was prepared similarly but the reaction mixture without incubation was heated in a boiling water bath for 5 min to denature the endoglucanase.

For preparing standard curve, 50 µl of glucose standard solution (5, 10, 15, 20 and 30 µmol/ml) was mixed with 0.5ml of 1% CMC and incubated similarly followed by addition of 1ml DNSA reagent, boiling, centrifuging and estimation of the color at 530nm. A blank was prepared with 50µl acetate buffer instead of glucose standards. The amount of sugar produced by enzyme reaction was calculated from its optical density using standard curve obtained from glucose standards.

4.3 Protein estimation

4.3.1 Lowry method

The protein content was estimated by the method of Lowry et al., 1951 as given below.

4.3.1.1 Reagents:

1. Reagent A :Reagent A was prepared by dissolving 0.5g of copper sulphate ($CuSO_4$. $5H_2O$) and 1g of sodium citrate in 100ml of deionised water. This solution can be kept indefinitely.

2. Reagent B: 20g of sodium carbonate (Na_2CO_3) and 4 g of sodium hydroxide (NaOH) were dissolved in 1 litre of deionised water to obtain reagent B.

3. Reagent C :Reagent C was freshly prepared just before use by adding 50 ml reagent B to 1ml of reagent A.

4. Reagent D: It was prepared by adding Folin Ciocalteau reagent and deionised water in 1:1 ratio just before use.

5. BSA standard: 5mg of BSA was dissolved in deionised water and made upto 5ml.

4.3.1.2 Procedure:

Various concentrations of the BSA standards (0.1 – 0.5 ml), and samples (0.2ml after appropriate dilution) were made upto 0.5ml with deionised water. To each of the tubes were added 2.5 ml of reagent C, mixed and allowed to stand for 5-10 minutes. Then 0.25 ml of reagent D was added, mixed and allowed to stand for 20 to 30 minutes. The absorbance was read at 700 nm against reagent blank. Standard curve was plotted with concentration of BSA on x-axis and optical density on y-axis. The protein concentrations of the samples were estimated

with the help of standard curve. The protein concentration in the solution was calculated and expressed in mg protein per ml of extract.

4.3.2 Spectrophotometric method

The eluent fractions of Gel filtration chromatography were monitored, by measuring the absorbance at 280nm against buffer solution. The absorbance is due to the aromatic amino acid residues of tyrosine and tryptophan.

4.4 Extraction procedure

4.4.1 Desalting of the extract:

About 6 g of Sephadex G-25 (30 x 3.1cm) was suspended in water and allowed to swell overnight. The swollen Sephadex was deaerated and the fine particles removed by decantation. The gel suspension was brought to 4°C and poured into a column, which was previously half filled with sodium acetate buffer (0.1M) and maintained at 4°C. The partially purified endoglucanase obtained from fractionation with 60% ammonium sulphate was applied to the column and eluted with 0.1M sodium acetate buffer. The eluents were collected in 10ml fractions and monitored at 280nm. The protein fractions were collected and pooled. The activity and protein content of the desalted endoglucanase were determined.

4.4.2 Sephadex G-100 chromatography

Gel filtration was performed on Sephadex G-100. About 3 g of gel was suspended in 100ml of water and allowed to swell for 48 hours. The swollen

Sephadex was deaerated and the gel poured into a glass column (45cm x 1.7cm), which was previously half filled with sodium acetate buffer (0.1M). As the gel was poured, the excess buffer was allowed to percolate gradually through the bed. The gel height was measured. The column was equilibrated with the same buffer, the flow rate and void volume noted and this column was used for further purification of the enzyme preparation.

4.4.3 Enzyme homogeneity tests:

The homogeneity of endoglucanase was tested by electrophoresis on polyacrylamide gel and chromatography on sephadex G100 column as described below.

4.4.3.1 Polyacrylamide gel electrophoresis

4.4.3.1.1 Principle: Vertical gel electrophoresis was conducted by the method of Laemmli (1970) with some change in sample preparation. It is based on the principles that, in the presence of 10% SDS and 2-mercaptoethanol, proteins dissociate into their sub units and bind large quantities of the detergent which mask the charge of the proteins and giving a constant charge to mass ratio so that the proteins move according to their molecular weight in an electric field. In this discontinuous buffer system, the separating (resolving gels) and stacking gels are made up in the electrode buffer, Tris-glycine. During electrophoresis, the leading ion is chloride while the trailing ion is glycine.

4.4.3.1.2: Preparation of reagents and gel:

1) Tris-HCl: 0.5M, pH 6.8: 6 g of Tris(hydroxymethyl)aminomethane was dissolved in deionised water and made upto 100ml with after adjusting the pH to 6.8.
2) Tris-HCl: 1.5M, pH 8.8: 27.23g of Tris(hydroxymethyl)aminomethane was dissolved in deionised water and made upto 150ml with after adjusting the pH to 8.8.
3) SDS 10%: 10 g of SDS was dissolved in 100 ml of deionised water.
4) APS 10%: Freshly prepared by dissolving 100 mg of APS in 1ml of deionised water to get 10% APS.
5) 1% bromophenol blue: 1 g of bromophenol blue was dissolved in 100 ml of water.
6) Acrylogel 30%: 30 g of acrylogel was dissolved in 100 ml of deionised water.
7) Coomassie blue R250 stain (1% in 10% acetic acid and 40% methanol): Prepared by dissolving 0.1g of Coomassie blue R250 in 50ml acetic acid, then added 200ml of methanol and 250ml of deionised water.
8) Preparation of sample buffer:

   The sample buffer was prepared by adding 1ml of Tris-HCl 0.5M, 0.8 ml of glycerol, 1.6 ml of 10% SDS, 0.4ml of 2-mercaptoethanol and 0.4ml of bromophenol blue to 3.8ml of deionised water.
9) Preparation of sample:

The crude extract was diluted thrice to get low concentration of protein. Then 0.1ml of this diluted sample was mixed with 0.3ml of sample buffer. Purified

extract was as such diluted by adding 0.2ml sample buffer to 0.1ml of extract in microfuge tube. Microfuge tubes containing crude and purified extracts separately were heated in boiling water bath for 4 minutes, cooled and kept frozen until used for investigation.

10) Preparation of Electrode buffer:

The electrode buffer was prepared by adding 9g of Tris base, 43.2g of glycine and 3g of SDS to 600ml of deionised water.

Working solution: For each electrophoretic run working solution was prepared by diluting 100ml of the electrode buffer to 500ml with deionised water.

11) Preparation of separating gel: 10%

Deionised water: 4.05ml

1.5M Tris-HCl: 2.5ml

10%SDS: 100µl

Acrylogel: 3.3ml

10% APS: 50µl

TEMED: 20µl

Running gel (10ml) of 10% was prepared by mixing 4.05ml of deionised water, 2.5ml of 1.5M Tris – HCl pH 8.8, 100 µl of 10% SDS and 3.3 ml of 30% acrylogel. Then it was degassed for more than 15 minutes at room temperature. After degassing, 20µl of TEMED was added and shaken well. To this 50µl of 10% APS was added and mixed well.

12) Preparation of stacking gel 4%

Deionised water: 6.1ml

1.5M Tris-HCl: 2.5ml

10%SDS: 100µl

Acrylogel: 1.33ml

10% APS: 50µl

TEMED: 20µl

Stacking gel (10ml) of 4% acrylogel was prepared by mixing 6.1ml deionised water, 2.5ml of 0.5M Tris – HCl, pH 6.8, 100µl of 10% SDS and 1.33ml of 30% acrylogel. Then it was degassed till no bubble was observed (15-20 minutes). To this 20µl of TEMED was added followed by 50 µl of 10% APS and mixed well.

4.4.3.1.3. Casting of discontinuous (Laemmli) Polyacrylamide Gel:

Comb was inserted in between the assembled gel sandwich of "Biorad" mini protein II electrophoresis plates. Mark was made on glass plate 1cm below the teeth of comb, this was the level to which the separating gel was poured and comb was removed. Prepared separating gel was poured immediately after adding TEMED and APS and overlaid with water slowly with steady even rate of delivery to prevent mixing. The gel was allowed to polymerize for 45 minutes. Over layer of water was removed and comb was placed in a tilted position. After adding TEMED and APS to the stacking gel, it was poured till the gel had covered the gap between the teeth of the comb. The comb was properly placed.

Gel was allowed to polymerize for 45 minutes. Comb was removed and gels were assembled on the inner cooling case.

4.4.3.1.4. Sample application and run

Electrode buffer (2 - 4°C) was poured into the chamber and about 10µl of the samples were applied per well of stacking gel. The cathode and anode of the gel assembly were then connected to the power supply. A constant voltage of 200 volts was then applied and kept constant throughout the run. When the dye front reached the bottom of the gel, power supply was switched off. The slabs were dismantled from the unit and allowed to cool.

4.4.3.1.5. Staining of gels

The gels from the glass plates were then removed carefully and stained with 0.1% coomassie blue R-250 dissolved in 10% acetic acid and 40% methanol for 30 minutes. Destaining was done with 7% acetic acid, with several changes to remove background dye.

4.4.3.2. Sephadex G100 chromatography:

To test the homogeneity of the purified sample, gel filtration chromatography was performed on Sephadex G100. The gel suspension was deaerated and poured into a glass column (1.7 x 45 cm) already half filled with deionised water. The gel was poured until the bed volume reached a height of 30 cm. The column was equilibrated with 0.1M sodium acetate buffer and the flow rate noted. The

sample was applied and eluted with 0.1M sodium acetate buffer. The eluent fractions were collected and tested for endoglucanase activity was under (4.2) and protein content by absorbance measurement at 280nm.

4.4.3.3 Absorption spectrum: Endoglucanase diluted in 0.1M sodium acetate buffer was measured for absorbance at various wavelengths from 200 to 400 nm using Spectonic Genesys 5. The UV absorption spectrum of green mussel endoglucanase was obtained by plotting optical density values on y-axis and absorbance on x-axis.

4.5 Characteristics of green mussel endoglucanase

4.5.1 Molecular weight determination

The molecular weight of pure endoglucanase was determined by SDS PAGE method [4.4.3.1]. Both high and low molecular weight markers were used for comparison as described below.

Procedure:

Electrode buffer (2 - 4°C) was poured into the chamber after assembling the gel in the chamber. To consecutive wells of the stacking gel were added 10 µl each of high molecular weight protein marker (Myosin, rabbit muscle: 205000, β-galactosidase, *E. coli*: 116000, Phosphorylase b, rabbit muscle: 97000, Fructose-6-phosphate kinase, rabbit muscle: 84000, Albumin, bovine serum: 66000, Glutamic dehydrogenase, bovine liver: 55000, ovalbumin, chicken egg, 45000, Glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle: 36000),

crude sample and purified sample and low molecular weight markers (Albumin, bovine serum: 66000, Ovalbumin, chicken egg: 45000, Glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle: 36000, Carbonic anhydrase, bovine erythrocytes: 29000, trypsinogen, bovine pancreas: 24000, trypsin inhibitor, soybean: 20000, α- Lactalbumin, bovine milk: 14200, aproptinin, bovine lung: 6500). The electrodes were connected to the power supply unit and a constant voltage of 200 volts was then applied. After electrophoretic run the gel was stained (4.4.3.1.5) and bands identified.

From the electrophorograms obtained, the relative mobility of each protein applied was calculated as per the equation

$$\text{Relative mobility} = \frac{\text{Distance of protein migration x Gel length before staining}}{\text{Gel length after destaining x Dist. of bromophenol blue migration}}$$

4.5.2 Effect of substrate concentration on endoglucanase activity

To study the effect of substrate concentration on endoglucanase activity assay was performed as in 4.2 with 30µl of endoglucanase and various concentrations of CMC (0.1-0.8 mg/ml) in a series of test tubes. Line Weaver Burk plot was drawn with reciprocal of substrate concentration (CMC) (1/S) on x-axis and reciprocal of velocity of reaction (1/v) on y-axis. From the graph y - intercept and slope were measured and Vmax and Km were calculated using Michaelis Menten equation, $V=\frac{V_{max}[S]}{[S] + K_m}$, where V=reaction rate, [S]=substrate concentration, K_m = Michaelis Menten constant (Measures enzyme substrate

affinity), K_m is the substrate concentration at half the maximal velocity, V_{max} = maximum rate

4.5.3 Effect of temperature on endoglucanase activity

To study the effect of temperature on endoglucanase activity, assay was performed as in 4.2 with 30µl of endoglucanase and 0.5ml of 1% CMC in a series of test tubes and incubated at various temperatures (-20°C, 0°C, 20°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, 100°C) for 30 minutes. The endoglucanase activity values (in terms of percentage of maximum) obtained at various temperatures was plotted on y-axis and the temperatures of incubation on x-axis. The optimum temperature was determined from the graph.

4.5.4 Effect of pH on endoglucanase activity

The effect of pH on endoglucanase activity was studied with McIlvaine and glycine - sodium hydroxide buffers as described below. The buffers were prepared as given by Dawson et al., (1986).

a) McIlvaine buffers (pH 2.6-7.6):

Citric acid monohydrate (0.1M): 21.01 g of citric acid monohydrate was dissolved in 1 litre of deionised water.

Disodium hydrogen phosphate (0.2M): 35.61 g of Na_2HPO_4 was dissolved in 1 litre of deionised water.

McIlvaine buffers (pH 2.6-7.6) were prepared by adding 0.1M Citric acid and 0.2M disodium hydrogen phosphate (Na_2HPO_4) as described in the Table 4.1.

Table 4.1: McIlavaine buffers

pH	0.1M Citric acid (in ml)	0.2M Na_2HPO_4 (in ml)
2.6	89.10	10.90
3.0	79.45	20.55
4.0	61.45	38.55
5.0	48.50	51.50
6.0	36.85	63.15
7.0	17.65	82.35
7.6	6.35	93.65

(pH 2.6-7.6)

b) Glycine – NaOH buffer (pH 8.6-10.0):

Glycine (0.2M): 4.03 g of glycine was dissolved in 300ml of deionised water.

Table 4.2: Glycine – NaOH buffers

pH	Volume of 0.2N NaOH (in ml)
8.6	2.0
9.0	4.4
10.0	16.0

(pH 8.6-10.0)

Glycine – NaOH buffer was prepared by adding different volumes of 0.2 N NaOH (Table 4.2) to 25 ml 0.2M Glycine solution and then making up the mixture to 100ml with deionised water.

The substrate was prepared in 1% concentration of various buffers (2.6-10.0). To study the effect of pH on the activity of endoglucanase, assay was performed as in 4.2 with 30µl of purified endoglucanase and 0.5ml of the substrate prepared with various pH solutions taken in a series of test tubes. The degree of enzymatic hydrolysis of the CMC at different pH was determined spectrophotometrically by measuring the absorbance at 530nm. Graph was plotted with pH values on x-axis and activity (in terms of percentage of maximum) on y-axis. The optimum pH was determined from the graph.

4.5.5 Determination of isoelectric point

The isoelectric point of endoglucanase was determined as detailed below according to the procedure given by Stroev and Makarova, 1989.

Principle: Proteins are amphoteric in nature that is they carry both positive and negative charges. The charge on a protein molecule determines the agglomeration of protein particles and their precipitation. The net charge is affected by pH of the medium. Each protein has a characteristic pH value at which the sum of positive and negative charges on the protein is zero or the molecule is electrically neutral. This is its isoelectric point i.e., at this pH proteins loose biological activity and deposit as precipitate especially in the presence of dehydrating agents like ethanol, acetone, and others.

Reagents:

(1) McIlvaine phosphate and glycine - sodium hydroxide buffers were prepared as given under [4.5.4]

(2) 96% Ethanol

Procedure: To 3 ml of buffer solutions (pH 3 to 9) taken in a series of test tubes were added 20 µl of purified enzyme and shaken well. The tubes were observed for cloudiness. Then 2 ml of ethanol (96%) was added to each of the tubes and cloudiness visually examined and compared. The turbidity was measured using a turbidometer. The results were expressed in terms of NTU.

4.5.6 Estimation of amino acids other than tryptophan

Amino acids were estimated according to the procedure of Ishida et al. (1981) with little modification.

4.5.6.1 Preparation of reagents:

1. Mobile phase buffers: The mobile phase buffers were prepared by mixing the reagents and adjusting to required pH have been given in Table 4.3.

2. OPA buffer: OPA buffer is prepared by dissolving the reagents (Table 4.4) in water and making up to 3 liters.

3. OPA reagent: OPA reagent was prepared by dissolving the reagents listed as per Table 4.5 in 500ml OPA buffer.

Table 4.3: Composition of mobile phase buffers for amino acid analysis

Reagents	Buffer A	Buffer B
Tri sodium citrate	58.80	58.80
Boric acid (ml)	-	12.40
Ethanol (99.5%)ml	210.00	-
Perchloric acid (60%) ml	50.00	-
4N NaOH (ml)	-	45.00
Final volume (l)	3.00	1.00
pH	3.59	9.21

Table 4.4: Composition of OPA buffer

Reagents	Quantity (g)
Sodium carbonate	122.10
Boric acid	40.75
K_2SO_4	56.40

Table 4.5: Preparation of OPA reagent

Reagents	Quantity
OPA	400 mg
99% Ethanol	7ml
2-mercaptoethanol	1ml
30% Brij-35 solution	0.75 ml

4. Sodium hypochlorite solution: 0.5ml of sodium hypochlorite solution was added to 100ml of OPA buffer.

5. Preparation of standard: 0.1ml of the aminoacid standard was made upto 1ml with 0.05M HCl.

6. Preparation of Sample: 1ml of purified enzyme extract containing (3mg protein) was pipetted into a heat sealable test tube into which 5ml of 6N HCl was added. Air was removed from the test tube by passing nitrogen, before it was sealed. Hydrolysis was carried out in hot air oven at 110°C for 24 hours. After the hydrolysis was over the test tube was broken open. The contents were removed quantitatively and filtered into a round bottom flask through Whatman Filter Paper No.42 the filter paper was washed 2-3 times with deionised water. The contents of the flask was flash evaporated at 50 - 60°C to remove traces of HCl, the process was repeated 2-3 times after adding deionised water. The residue was made upto 2 ml with 0.05M HCl. The sample thus prepared was filtered again through a 0.45μm membrane filter.

4.5.6.2 Procedure: 20 μl of the sample was injected into a Shimadzu HPLC-LC 10AS (This system of aminoacid analyzer consists of column packed with a strongly acidic cation exchage resin (i.e.) styrene divinyl benzene copolymer with sulfinic group. The column is Na type i.e. ISC-07/ S1504 Na with a length of 19 cm and diameter 5mm. The mobile phase of the system consists of two buffers A and B (Table 4.3). The aminoacids were eluted from the column by stepwise elution i.e. acidic aminoacids first followed by neutral and then basic aminoacids.

The aminoacid analysis was done with non-switching flow method and fluorescence detection after post column derivatization with o-phthalaldehyde. In the case of proline and hydroxyproline imino group was converted to amino group with sodium hypochlorite. Aminoacid standard was also run to calculate the concentration of sample aminoacid depending on the standard chromatogram. The amino acid content was calculated and expressed as percentage mg/100mg protein.

4.5.7 Estimation of tryptophan

Tryptophan in the enzyme was estimated by the method of Sastry and Tammuru (1985) with some modification in sample and reagent preparation. About 1ml sample was hydrolysed with 3ml of 5% NaOH at 110°C for 24 hours in a sealed tube filled with pure nitrogen. The hydrolysate was neutralized to pH 7.0 with 6N HCl using phenolphthalein indicator. The volume was made up to 25ml with deionised water. This was then filtered through Whatman filter paper No.1 and the filtrate was used for estimation of tryptophan. Tryptophan content in the sample was determined as per the procedure explained in Section 4.5.7.1 below.

4.5.7.1 Preparation of standard and standard curve

Standard solution of Tryptophan containing 10μg/ml was prepared in 0.1N hydrochloric acid. 2.5% sucrose and 0.6% thioglycolic acid each at 0.1ml level were successively added to test tubes containing 4ml of 50% sulphuric acid. The test tubes were shaken and incubated at 45-50°C for 5 min and cooled.

Standard solution of tryptophan ranging from 0.05 – 0.5ml (0.5 to 5μg) was added and mixed. The volume was made upto 5ml with 0.1N HCl and was kept for 5 minutes for the development of full colour. The absorbance was measured at 500nm against a reagent blank. A standard calibration curve was prepared for deducting the amount of tryptophan. Tryptophan content was finally expressed as mg/100 mg protein.

4.5.8 Effect of certain inorganic ions and heavy metals on the activity of endoglucanase

10mM concentration of various anions F^-, Cl^-, I^- and Br^- and cations Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, Sn^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+}, Ba^{2+}, Ca^{2+}, Pb^{2+}, Cd^{2+}, Hg^{2+}, Ag^+ were prepared from the salts of these ions. To determine the effect of these ions on the endoglucanase activity, the assay was conducted as in 4.2 with 50μl of endoglucanase, 0.5ml of 1% CMC dissolved in 0.1M sodium acetate buffer and 1 ml of 10mM concentration of each ions taken in a series of test tubes. The degree of enzymatic hydrolysis of the CMC in presence of each ion was determined spectrophotometrically by measuring the absorbance at 530nm.

4.5.9 Immobilisation of endoglucanase

Endoglucanase was attempted immobilization with two solid phases viz. polyacrylamide gel and chitosan as described below.

4.5.9.1 Immobilisation of endoglucanase on Polyacrylamide gel

0.5ml of the enzyme solution was mixed with 3ml of 0.1M sodium acetate buffer pH 5.0, 3ml of acrylogel containing 30% acrylamide and 2.67% N, N -

methylenebisacrylamide. To this was added 50 µl of 10% APS and 20µl TEMED and mixed well. The mixture was kept for 15 min to gel. The gel was lyophilized to get immobilized endoglucanase. The immobilized endoglucanase was stored at 0°C and activity studied.

4.5.9.2 Immobilisation of endoglucanase on chitosan

0.5g chitosan was dissolved in 50ml of 1% acetic acid of pH 5.0. To 10 ml of this solution, 0.5ml of endoglucanase was added and mixed in a vortex mixer at intervals and kept for 2 hours at 0-4°C. The solution was neutralized with 0.1N sodium hydroxide. Chitosan precipitates. The precipitate was centrifuged at 6000 g in a refrigerated centrifuge for 10 minutes. The precipitate was washed free of salt and kept at 0-4°C. The activity was studied.

5. RESULTS AND DISCUSSION

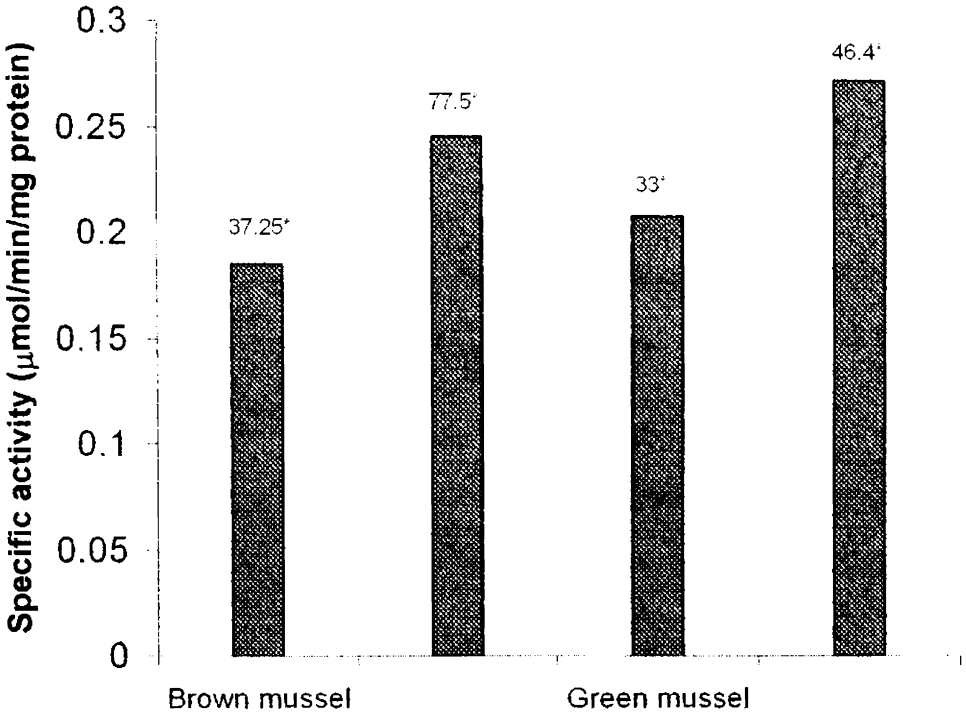
5.1 Endoglucanase activity in mussels.

5.1.1 Distribution of endoglucanase activity with size and species of mussel.

In the early stages of study, *Perna viridis* (green mussel) and *Perna indica* (brown mussel) each of two different sizes were brought from Central Marine Fisheries Research Institute hatchery and processed. 1g of the digestive gland was homogenized in 0.1M acetate buffer, pH 5.0 with 0.04% Tween 20. All operations were carried out at 4°C. The samples were centrifuged at 6000 g for 10 min and the clear extract assayed for endoglucanase activity as per the method described under 4.2. The protein concentration was estimated by Lowry method (4.3.1). The specific activity thus calculated was compared and it was found that green mussel produced (nearly 10% more) endoglucanase when compared with brown mussel. The results are represented in Figure 5.1.

Cellulases along with other carbohydrases have been identified in the digestive gland of *Perna viridis* (Toe & Sabapathy, 1990). No studies have been reported on cellulases of *Perna indica*. The results obtained in the present study show that *Perna viridis* is a better cellulose producer when compared with *Perna indica*. This could be correlated with the fact that *Perna viridis* has the greatest growth rate among the different species of mussels studied (Shafee, 1979) which in turn can be related to feeding habit that they prefer an abundance of phytoplankton in the growing areas (Chatterji et al., 1984).

Figure 5.1 Comparison of specific activities of *Perna viridis* and *Perna indica*



* indicate average of the total meat weight of the mussel (in g)

Considering the abundance of green mussel along the Kerala coast the study was continued with isolation and purification of endoglucanase from green mussel.

5.1.2 Distribution of endoglucanase in green mussel, *Perna viridis*.

Fresh mussel were dressed and processed to get the hepatopancreas and whole meat. They were separately minced with double the volume of 0.1M acetate buffer, pH 5.0 with 0.04% Tween 20. All operations were carried out at 4°C. The samples were centrifuged at 6000 g for 10 minutes and the clear extract assayed for endoglucanase activity. The activity values obtained are shown in Table 5.1. The endoglucanase activity in the hepatopancreas of green mussel is 56.2 % more when compared with that in the whole meat indicating it as a better source of the enzyme.

Though very high cellulase activity has been identified in the digestive gland of *Perna viridis* (Toe & Sabapathy, 1990), no comparison has been made with that in the whole meat. Endoglucanase has been purified from frozen whole meat as well as the digestive gland of blue mussel, *Mytilus edulis* and it has been reported that the endoglucanase activity in the crude extract is low when compared to that in the digestive gland (Xu et al., 2000). The studies thus show that endoglucanase is localized in the digestive gland. Moreover, crystalline style (which contains the digestive enzymes) present in the digestive gland (Kristensen, 1972) is absent in animals that have not fed recently.

Table 5.1 Distribution of Endoglucanase activity in *Perna viridis*

Perna viridis	**Activity μ moles/min/mg protein**
Hepatopancreas	113.00
Whole meat	49.50

As the endoglucanase concentration of hepatopancreas was appreciable an effort was made to purify the endoglucanase from the hepatopancreas of green mussel and study some of its characteristics and properties.

5.1.3 Determination of optimum ammonium sulphate concentration

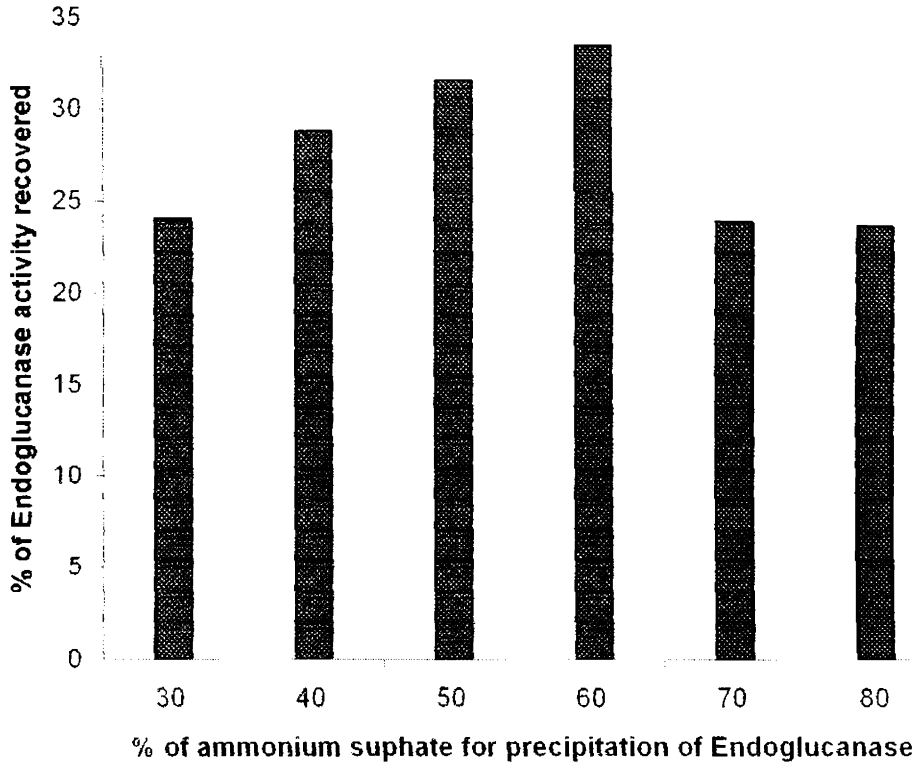
About 60 g of mussel was homogenized in 120 ml of acetate buffer (0.1M, pH 5 with 0.04% Tween 20) and centrifuged at 6000g at 4°C for 10 minutes. To each 10 ml of the extract, ammonium sulphate was added to get saturations of 30%, 40%, 50%, 60%, 70% and 80%. The precipitate was centrifuged at 6000g, 4°C for 15 minutes. Both the precipitate and supernatant were dialyzed using a dialyzing membrane and 0.1M acetate buffer. The endoglucanase activity and protein concentration were determined as in 4.2 and 4.3.1 respectively. The results show that though maximum protein gets precipitated at ammonium sulphate saturation 70%, maximum endoglucanase activity is obtained at 60% saturation. A maximum of 33.49% recovery only could be obtained. The percentage of endoglucanase activity recovered corresponding to various ammonium sulphate saturations are shown as bar diagram in Figure 5.2.

5.2 Purification of green mussel endoglucanase

5.2.1 Preparation of crude extract

The mussel in live condition was brought from Tikkodi. The meat was shucked from 40 - 50 animals, the hepatopancreas excised and the same was used as a source of endoglucanase. The hepatopancreas excised were chilled and maintained at 0°C for the study. All remaining operations were done between

Figure 5.2 Percentage of Endoglucanase activity obtained at various ammonium sulphate saturations



0°C and 4 °C unless otherwise specified. About 50g of digestive gland was used in each batch of enzyme isolation. The hepatopancreas was then homogenized in 100ml of 0.1M sodium acetate buffer. The contents were centrifuged at 6000g, 4°C for 15 minutes. The endoglucanase activity was estimated according to the method given by Wood and Bhat (1998) (4.2) and protein content by Lowry's method 4.3.1. Specific activity of the crude extract was calculated to be 0.297 μmole glucose /min /mg protein.

5.2.2 Partial purification of green mussel endoglucanase

All operations were carried out at 4°C unless otherwise specified. To the crude enzyme preparation ammonium sulphate was added to give a final ammonium sulphate concentration of 30% saturation at 0°C, stirred well and kept overnight in a cold room. The protein precipitated was collected by centrifugation in a refrigerated centrifuge at 6000 g for 15 minutes. The pellet was discarded and in the supernatant more solid ammonium sulphate was added to attain 60% saturation at 0°C and kept overnight. The precipitate composed of endoglucanase collected by centrifuging at 4°C as before and was dissolved in 20ml of 0.1M sodium acetate buffer to give partially purified endoglucanase.

Endoglucanase has been precipitated with 75% ethanol (Saha, 2003) and with 1M formic acid and heat treatment (90°C for 10min) (Xu et al., 2000), however fractional precipitation with ammonium sulphate is widely adopted for partial purification of endoglucanase (Saleem et al., 2004; Moriyoshi et al., 2003; Lin & Stutzenberger, 1995; Murashima et al., 2002). The precipitation technique

employed in turn depends on the endoglucanase of interest that is to be purified. In other words by employing different precipitation methods, one or more endoglucanases of varying degrees of purity could be isolated. This could be supported by the fact that both heat sensitive 70KDa and heat resistant 20KDa endoglucanases were identified in the crude extract of *Mytilus edulis* and one of them, namely the 20KDa endoglucanase was purified by heat treatment (Xu et al., 2000).

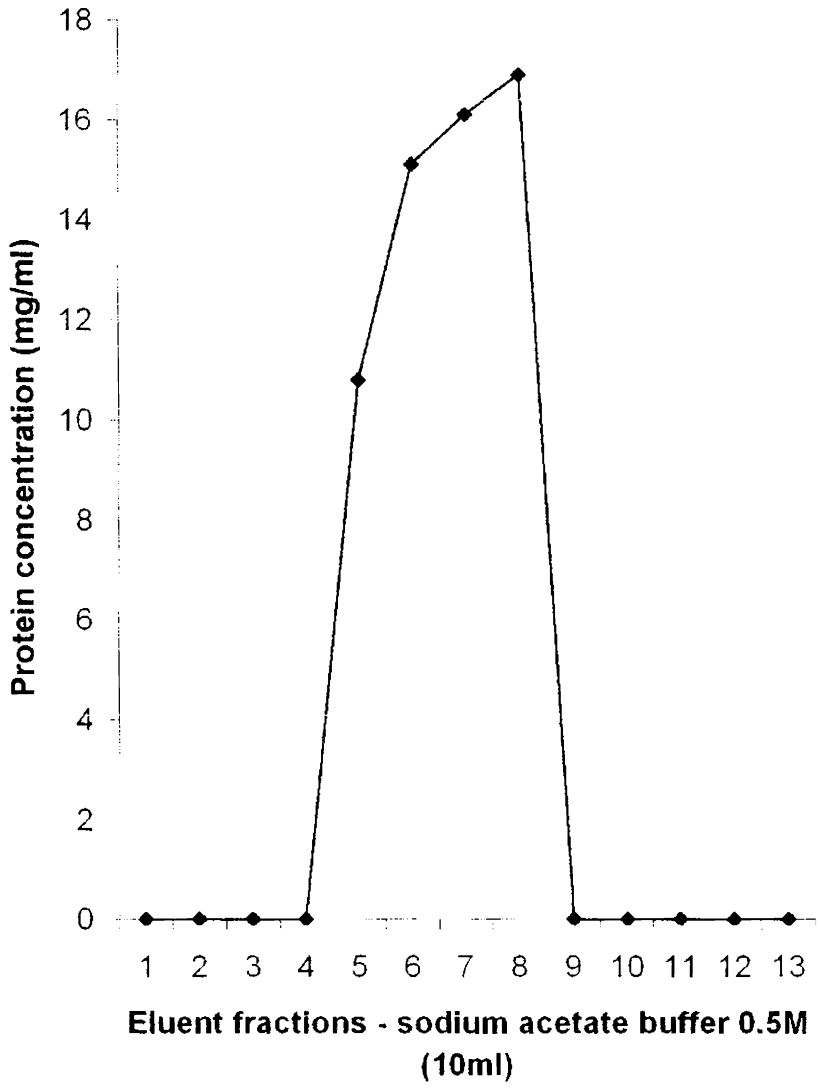
5.2.3 Desalting of proteins

To the G-25 packed column (9cm height) equilibrated with sodium acetate buffer (0.1M) the partially purified endoglucanase from ammonium sulphate fractionation was applied and eluted with 0.1M sodium acetate buffer. The flow rate was noted as 1 ml/min. The eluent was collected in 10 ml fractions and measured for absorbance at 280nm. The elution pattern at 280nm is shown in Figure 5.3. Endoglucanase activity and protein concentration of this solution was assayed as given in 4.2 and 4.3.1 respectively.

5.2.4 Sephadex G-100 chromatography

The protein fractions were pooled and concentrated to 20ml by adding 5 g of dry Sephadex G25. The desalted endoglucanase solution after concentration was fed to a column of Sephadex G-100 (1.7 x 45 cm, Pharmacia) which was washed and equilibrated with 0.1 M sodium acetate buffer and maintained at a flow rate of 3ml/15 min. 10ml of the desalted endoglucanase was fed to the column

Figure 5.3 Desalting of the protein on Sephadex G 25 column



without disturbing the bed. The sample was allowed to drain into the gel bed and initially elution was performed with 70ml of 50mM imidazole buffer (sodium acetate buffer containing 50mM imidazole) to remove less strongly bound proteins and then with sodium acetate buffer containing 50mM EDTA (endoglucanase gets eluted) until the eluent was almost free from UV absorbing materials. The eluent was collected in 3 ml portions and absorbance measured at 280nm in a UV spectrophotometer. The EDTA eluents were qualitatively identified by pink coloration with murexide. A suitable aliquot (50μl) from each of the fractions were analysed for enzyme activity. Most of the unwanted proteins get eluted with 50mM imidazole buffer. The strongly bound endoglucanase is retained in column and is eluted with 50mM EDTA. The elution pattern of green mussel endoglucanase, so obtained is shown in Figure 5.4.

There was only one activity peak suggesting one molecular species of endoglucanase. This peak was eluted in two fraction collection units corresponding to elution volume from 42 ml to 48ml. The eluent corresponding to this volume showed high endoglucanase activity. No other protein peaks were found to contain endoglucanase activity. A specific activity of 29.76 μmole glucose /min /mg protein was obtained. The summary of purification of green mussel endoglucanase is shown in Table 5.2.

The specific activity of green mussel endoglucanase increased from 0.297 units for crude to 2.75 units for desalted enzyme and to 29.76 units for the endoglucanase obtained after streamlined chelation on Sephadex G100 as a

Figure 5.4 Elution pattern of the desalted proteins on Sephadex G 100 column with 0.5M imidazole and 0.5M EDTA buffers

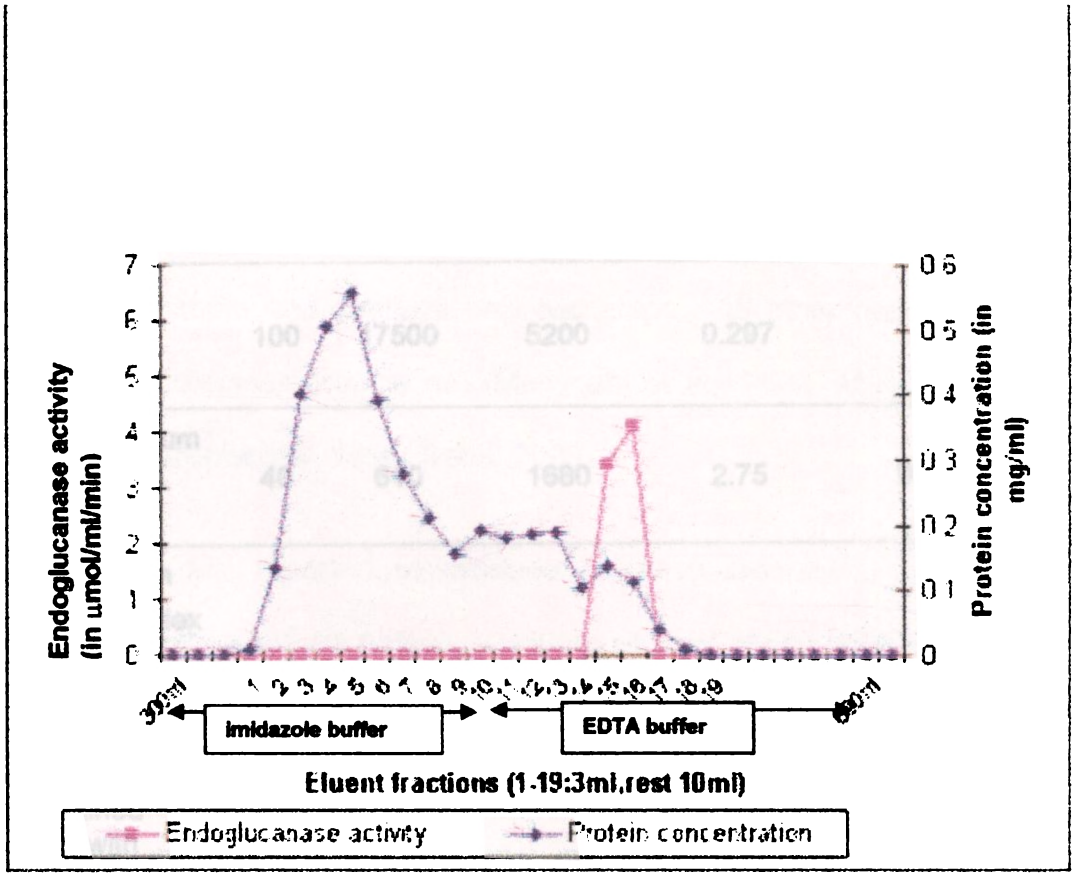


Table 5.2: Purification table for the green mussel Endoglucanase

Stage of purification	Fraction Vol. (ml)	Total protein (mg)	Total activity (μmol/ml/min)	Specific activity (μmol/min/ mg protein)	Purification (fold)	Recovery (%)
Crude	100	17500	5200	0.297	1	100
60% ammonium sulphate fractionation	40	610	1680	2.75	9.25	32
Concentration with Sephadex G25 & chromatography on Sephadex G 100, streamlined elution with imidazole and EDTA buffers	12	3.024	90	29.76	100.2	1.7

result of extraction with acetate buffer, ammonium sulphate fractionation, desalting and Sephadex G100 chromatography with imidazole and EDTA buffers. By this method endoglucanase can be 100 times purified with an overall yield of 1.7%.

The yield and purification fold of some endoglucanases are listed in Table 5.3. *Perna viridis* endoglucanase purification method adopted in the present study is relatively very simple and employs only two steps. All other methods employ more than one chromatographic run (Moriyoshi et al., 2003; Murashima et al., 2002; Lin & Stutzenberger, 1995; Saha, 2003).

Microorganisms are reported to produce cellulase isoenzymes (Table 2.2). *Humicola insolens*, a soft rot fungus, produces at least seven different cellulases (Schulein et al., 1993). *Aspergillus niger* produced four different endoglucanases while *Penicillium notatum* enzyme preparation gave three cellulases (Pettersson, 1969). *Rhizopus oryzae* isolated from the soil produced two extracellular endoglucanases (RCE1 and RCE2) in culture medium. The RCE1 and RCE2 were purified to 109 fold and 103.1 fold respectively with a recovery of 10.9% and 3.1% of the initial activity (Murashima et al., 2002). Three different endoglucanases of molecular weight 70 KDA, 45 KDa and 20 KDa have been identified in the extracts from *Mytilus edulis* digestive gland (Xu, 2002). By separate purification methods two different molecular species of endoglucanases have been identified in *Trichoderma viridae* (Berghem et al., 1976).

Table 5.3 Comparison of yield and purification fold of Endoglucanases from different sources

Source of endoglucanase	Yield (%)	Purification Fold
Neisseria sicca SB (Moriyoshi et al., 2000)	0.43	18.3
Rhizopus oryzae (Murashima et al., 2002)	3.1	103.1
Thermomonospora curvata (Lin & Stutzenberger, 1995)	2.8	389
Mucor circinelloides (Saha, 2003)	3	408
Green mussel *(Perna viridis)*	1.7	100.2

These reports show that the endoglucanase acitivity in the crude sample is the contribution of all different molecular species of endoglucanase present and that the contribution of each of these towards the total activity is very low. This could be the reason for the low yield (3% or less) of endoglucanases obtained when purified to homogeneity (Table 5.3).

5.3 Homogeneity of the purified green mussel endoglucanase

The high activity fraction of the green mussel endoglucanase obtained after Sephadex G 100 column chromatography with 50mM imidazole and 50mM EDTA buffers was then subjected to gel electrophoresis on polyacrylamide gel. The electrophoresis pattern of the diluted crude sample and the purified endoglucanase sample are shown in Plate 5.1. One of the bands on the crude sample (indicated by arrow) corresponds to the endoglucanase band.

3ml of the most active fractions from Sephadex G100 chromatography was applied to a fresh column of Sephadex G100 (45 x 1.7cm, Pharmacia) equilibrated with 0.1M sodium acetate buffer. The column was then eluted with the same buffer at a flow rate of 10ml/hour. The eluent fractions were collected in 5ml fractions and optical density of the eluents was monitored at 280nm. The elution pattern obtained is shown in Figure 5.5. There was only one protein peak, which correspond to the endoglucanase peak.

The absorption pattern and maxima of the purified endoglucanase protein was determined by scanning the optical density of the protein in the wavelength range

Plate 5.1 Gel electrophoresis pattern showing the crude sample (left) and the purified green mussel Endoglucanase (right)

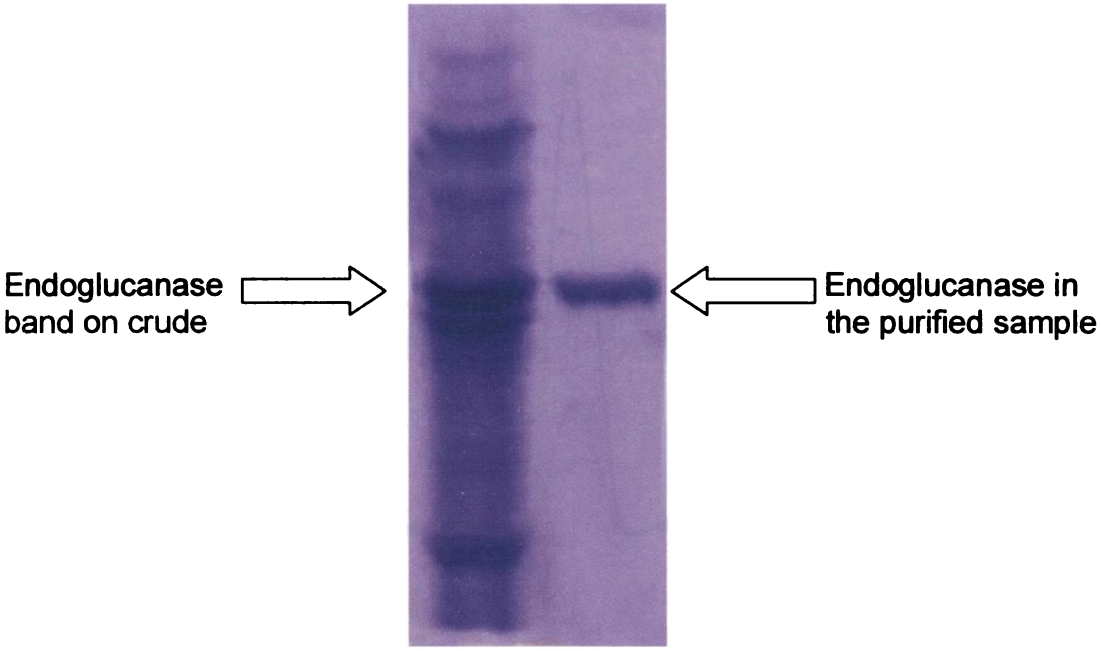
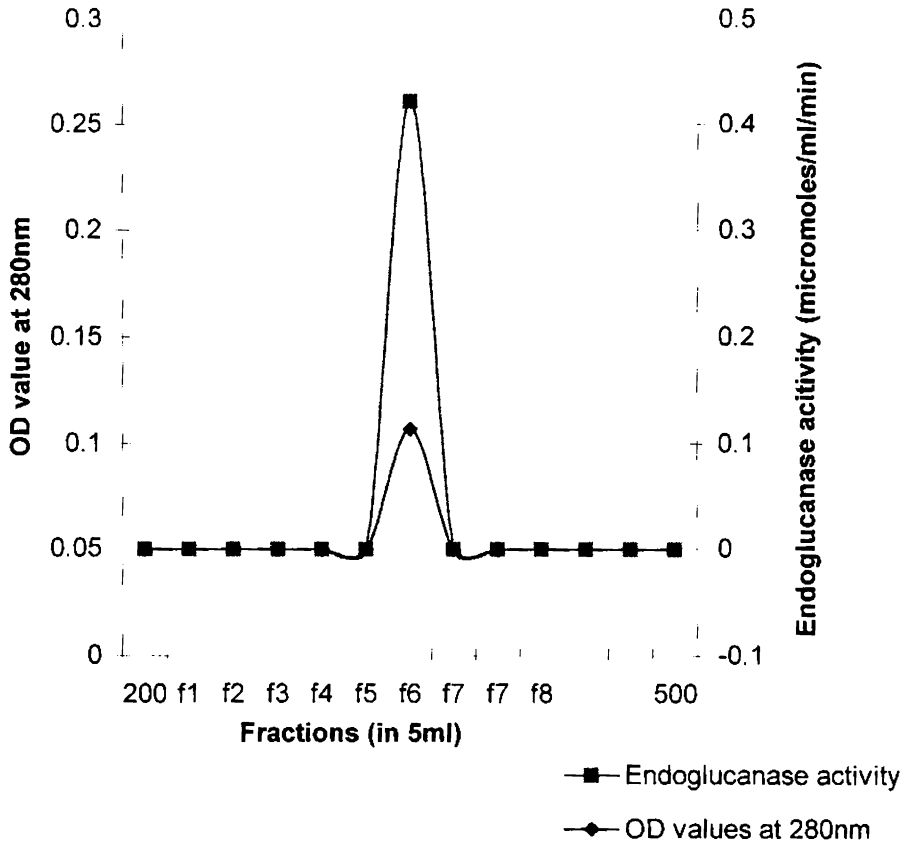


Figure 5.5 Gel filtration chromatography of the purified green mussel Endoglucanase on Sephadex G 100 column



from 200 to 400nm. The absorption spectrum obtained showed two peaks. The one at 280nm is indicative of the protein nature of endoglucanase. The nature of the group for the absorption maxima at 310nm is beyond the scope of the study. The absorption spectrum of pure endoglucanase protein obtained is shown in Figure 5.6.

From G100 column chromatography and SDS PAGE experiments the preparation of green mussel endoglucanase was found to be homogenous. Further confirmations in homogeneity could not be carried out due to lack of analytical ultracentrifuge.

The method of purification is thus relatively more efficient and cheap. The hepatopancreas being a waste product during processing makes it probably the cheapest source.

5.4 Estimation of molecular weight of the purified green mussel endoglucanase

Molecular weight of the purified green mussel endoglucanase was estimated by SDS PAGE method. In comparison with both high and low molecular weight markers endoglucanase gave a band between 55KDA and 45KDa for high molecular weight markers and between 66KDa and 45KDa for low molecular weight markers. The electrophoretic pattern of high molecular weight markers with pure endoglucanase is shown in Plate 5.2 and that of low molecular weight markers with pure endoglucanase is shown in Plate 5.3.

Figure 5.6: UV absorption spectrum of the pure Endoglucanase protein

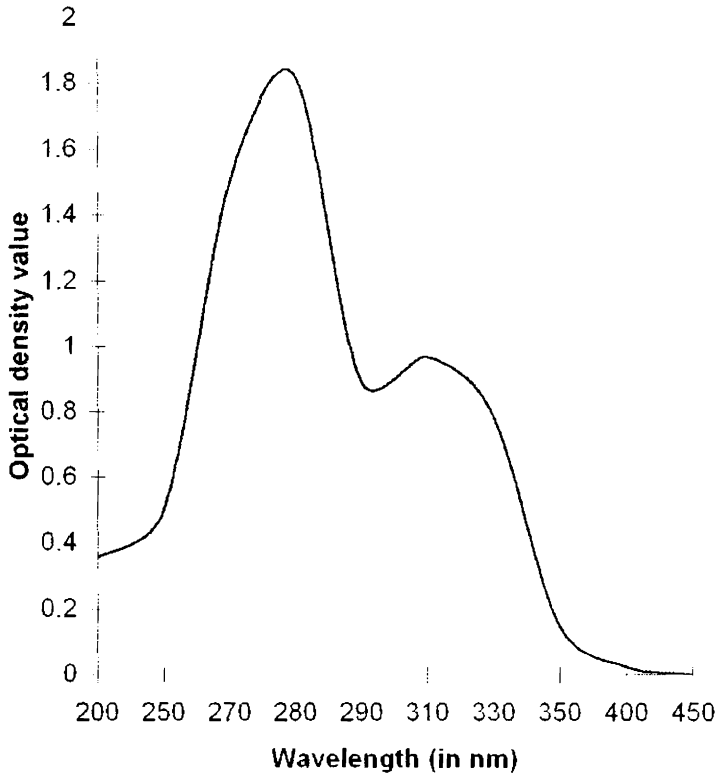


Plate 5.2 Gel electrophoresis pattern showing high molecular weight markers (left), crude sample (middle) and the purified green mussel endoglucanase (right)

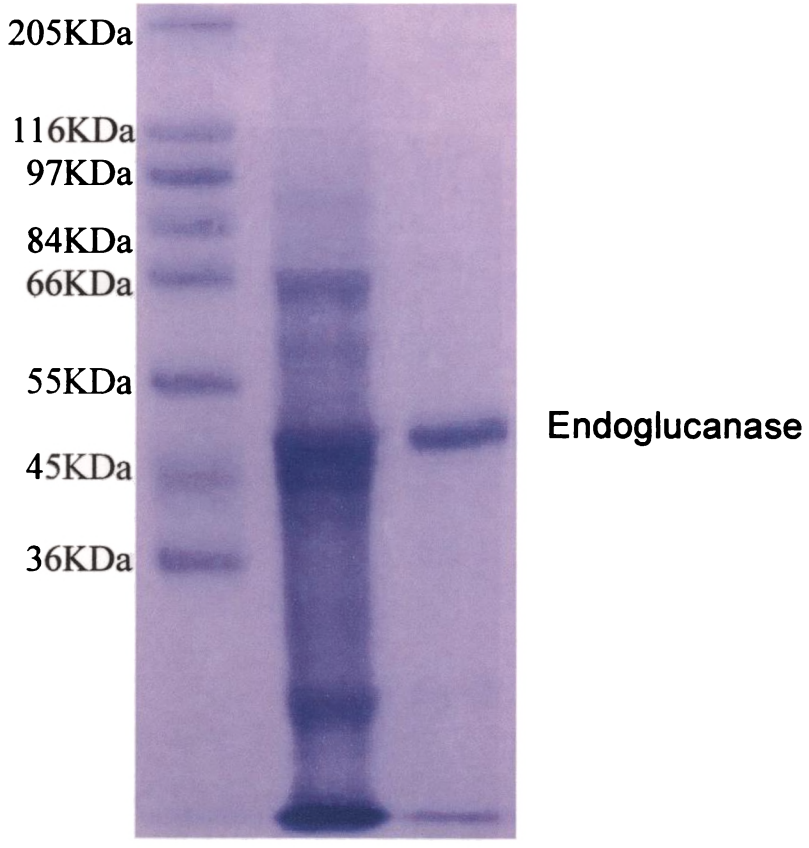
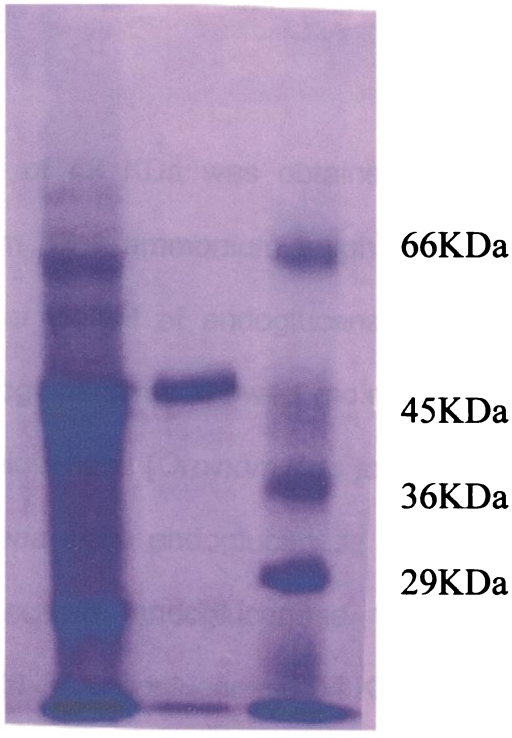


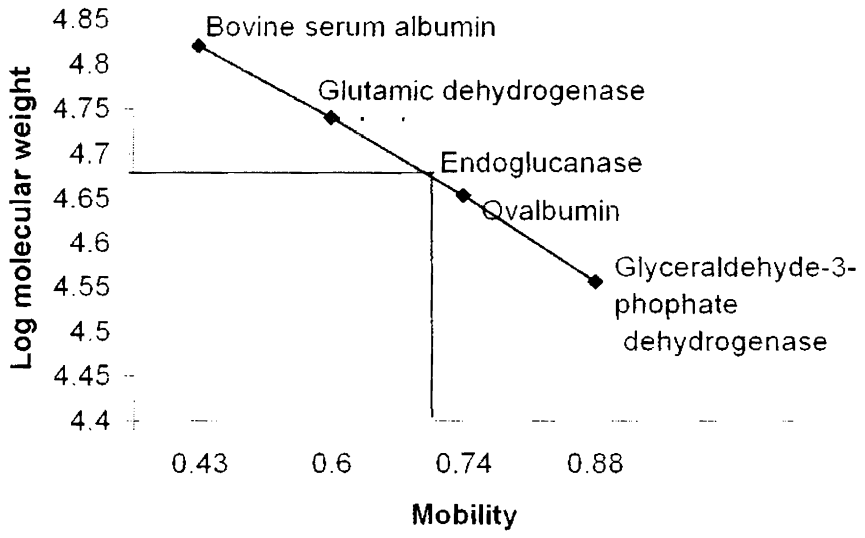
Plate 5.3 Gel electrophoresis pattern showing crude sample (left), purified green mussel endoglucanase (middle) and low molecular weight markers (right)



The low molecular weight markers diffused at a faster rate and all bands were not clear. The experiment could not be repeated due to non-availability of enough markers. However with high molecular weight markers all bands were clear and the mobility of each of the proteins was calculated using the formula given under 4.5.1. The mobility was plotted against log molecular weight to get a straight-line graph and the graph obtained is shown in Figure 5.7. From the graph, the molecular weight of the isolated green mussel endoglucanase was determined.

A molecular weight of 48 KDa was obtained for the purified green mussel endoglucanase (from 60% ammonium sulphate precipitation). Studies have shown that molecular weight of endoglucanase varies widely from source to source. Some endoglucanases showed molecular weight greater than that of *Perna viridis* endoglucanase [*Orpinomyces joyonii* endoglucanase 58 KDa (Ye et al., 2001), *Neisseria sicca* endoglucanase 50 KDa (Moriyoshi et al., 2003), *Thermomonospora curvata* endoglucanase 100 KDa (Lin & Stutzenberger, 1995), *Bacillus* sp. N6-27 endoglucanase 94 KDa (Tian & Wang, 1998), *Bacillus* endoglucanase 86KDa (Hakamada et al., 1997)] while certain other showed lower molecular weight [*Bacteroides succinogenes* endoglucanase 45 KDa (Groleau & Forsberg, 1983), *Rhodotorula glutinis* KUJ 2731 endoglucanase 40 KDa (Oikawa et al., 1998), *Bacillus* strains CH43 and HR68 endoglucanases 40KDa (Mawadza et al., 2000), *Mytilus edulis* endoglucanase 19 KDa (Xu et al., 2001)]. Thermophilic anaerobic bacterial endoglucanase showed two enzyme systems with molecular weights of 91 and 99 KDa (Creuzet & Frixon, 1983) while

Figure 5.7 Estimation of molecular weight of green mussel Endoglucanase by SDS PAGE.



Clostridium thermocellum endoglucanase showed molecular weight in the range 83 KDa - 94 KDa (Ng & Zeikus, 1981). Thus endoglucanases show wide variations in molecular weight not only between sources but also within the species. Cellulases released during germination of *Dictostelium discoideum* spores gave a molecular weight of 136 KDa and 69 KDa (Jones et al., 1979). Both low and high molecular weight endoglucanases (12500 and 50000 $\pm$ 2000) have been derived from cultures of *Trichoderma viridae* by separate purification procedures (Berghem et al., 1976).

5.5 Aminoacid compostion of the purified green mussel endoglucanase

The aminoacid composition of green mussel endoglucanase obtained is shown in Table 5.4. The aminoacid composition is calculated and expressed as percentage.

In the purified green mussel endoglucanase, glycine content was found to be the highest (12.5%), followed by proline (11.1%), aspartic acid (9.9%) and alanine (9%). Endoglucanase IV of *Trichoderma reesei* and Endoglucanase V of *Humicola insolens* (Davies et al, 1993) showed highest percentage for glycine viz 10.76% and 12.2 % respectively when compared with Endoglucanase C of *Clostridium cellulolyticum* and Cel 7b of *Humicola insolens*, in which alanine and leucine were the richest aminoacids (11.7% and 8.23% respectively).

The aminoacid composition of the purified green mussel endoglucanase showed nearly 70% similarity with that of Endoglucanase V of *Humicola insolens* (Davies

Table 5.4 Aminoacid composition (in mg %) of the purified green mussel Endoglucanase

Aminoacid	mg/100mg protein
Asp	9.9
Thr	6.1
Ser	7.1
Glu	8.1
Pro	11.1
Gly	12.5
Ala	9
Cys	2.2
Val	2.9
Met	1.1
Ile	3.9
Leu	2.6
Tyr	6.01
Phe	2.7
His	0
Lys	7.69
Arg	4.6
Trp	2.5

et al., 1993). Aminoacid histidine was not detected in the purified green mussel endoglucanase. However 1-2% of histidine is present in several endoglucanases (Saloheimo et al., 1997; Fierobe et al., 1993).

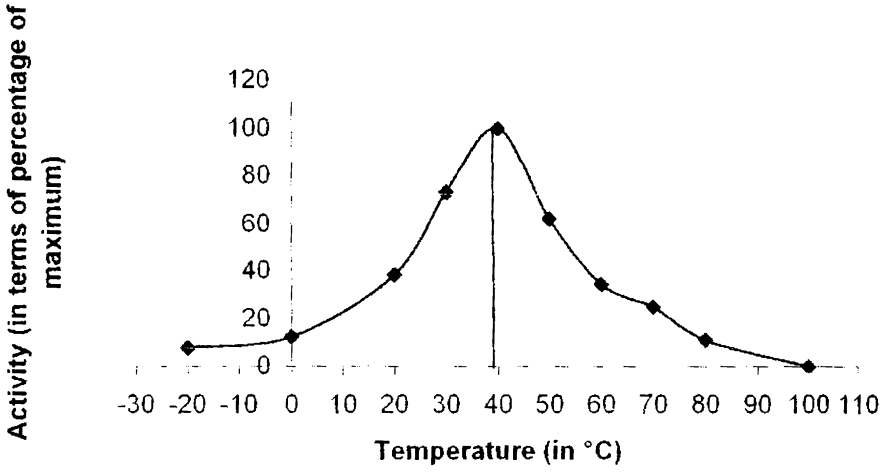
According to Hatch (1965) the residues Lys, Arg, Asp, Thr, Ser and Glu are considered to be polar aminoacids and Pro, Val, Met, Ile, Leu, and Phe as non-polar aminoacids. The ratio of the sum of polar aminoacid residues to non-polar aminoacid residues was found to be 1.79. This ratio varied between 1.5 and 2.0 for several endoglucanases (Davies et al., 1993; Saloheimo et al., 1997; Fierobe et al., 1993). This explains the hydrophilic nature of endoglucanases and the requirement of nucleophilic water molecules to initiate the hydrolysis of glycosidic bonds of the substrate (Sinnott, 1991; Mc Carter & Withers, 1994).

Aminoacid composition shows wide variations among glycoside hydrolase families and based on the aminoacid sequence, Henrissat has classified cellulases under several families (Families 5, 6, 7, 8, 9, 10, 12, 44, 45, 48, 61 and 74). Determination of aminoacid sequence of the purified green mussel endoglucanase is beyond the scope of the study.

5.6 Effect of temperature on the activity of green mussel endoglucanase

Figure 5.8 shows the variation of green mussel endoglucanase activity with temperatures from 0 to 100°C. The purified green mussel endoglucanase showed maximum activity at 40°C. The relative activity was found to rapidly decrease towards lower and higher temperatures. The endoglucanase activity at

Figure 5.8 Effect of temperature on the activity of Green mussel Endoglucanase



the optimum temperature (40°C) is 70% more when compared to that at 60°C or 15°C. The reproducibility of this characteristic has been confirmed using different substrate and enzyme concentration respectively.

The optimum temperature of green mussel endoglucanase was identical to that obtained for the fungus *Orpinomyces joyonii*, cloned in *Escherichia coli* (Ye et al., 2001). The endoglucanases of termite *Coptotermes formosanus* (Nakashima & Azuma, 2000), alkalothermophilic actinomycete (George et al., 2001) and psychrotrophic yeast *Rhodotorula glutinis* KUJ 2731 (Oikawa, 1998) exhibited optimum activity at 50°C. Endoglucanases of four strains of *Bacillus*, namely KSM-S237 (Hakamada et al., 1997), N6-27 (Tian & Wang, 1998), CH43 and HR68 (Mawadza et al., 2000) gave optimum temperatures of 45°C, 55°C, 65°C, and 70°C respectively. It is seen that the green mussel endoglucanase is active in the range of -20°C to 80°C with varying rate of cellulolysis. This is indicative of the fact that the frozen green mussel has the ability to retain 8-10% of the relative endoglucanase activity. Endoglucanase has been isolated and purified from frozen mussel *Mytilus edulis* (Xu et al., 2000) and was found to retain 50-60% original activity at 0°C. On the other hand, endoglucanase from *Clostridium thermocellum* gave an optimum temperature of 62°C (Ng & Zeikus, 1981) indicating the thermostability of that endoglucanase. In general it is seen that endoglucanases show optimum activity in the temperature range 45 – 55°C (Murashima et al., 2002; Ding et al., 2002; Ye at al., 2001).

The green mussel endoglucanase is mesophilic in nature. The green mussel lives in the mesophilic condition along the east and west coast of India. On the east coast it occurs as small beds along Chilka lake, Vishakapatnam, Kakinada, Madras, Pondicherry, Cuddalore, Porto Novo, and Port Blair. On the west coast extensive beds occur along Quilon, Alleppey, Cochin to Kasargode, Mangalore, Karwar, Goa, Bhatia creek, Malvan, Ratnagiri and Gulf of Kutch (Kuriakose, 1980).

5.7 Effect of pH on the activity of green mussel endoglucanase

The effect of pH on endoglucanase activity studied with McIlvaine phosphate buffer is shown in Figure 5.9. The green mussel endoglucanase activity was optimum at pH 5 when incubated at 40°C for 30 min. The pH optima obtained for endoglucanase from green mussel is identical to that reported for *Humicola insolens* and *Humicola grisea* (Hayashida et al., 1988) and alkalothermophilic actinomycete (George et al, 2001). *Mytilus edulis* endoglucanase showed maximum activity at pH of 4.6 (Xu et al, 2000). The green mussel endoglucanase was active over a wide pH range 4-7. More than 80% of the optimum activity is exhibited in the pH range 4-5.5, similar to the pH activity curves of 1, 4-β-glucan-glucanohydrolase of *Trichoderma reesei* (Ülker & Sprey, 1990) and endoglucanase of *Mytilus edulis* (Xu et al., 2000). However it is seen that the endoglucanase of *Mucor circinelloides* retained 80% activity at a wider pH range of 3-8 (Saha, 2003).

Figure 5.9 Effect of pH on the activity of Green mussel Endoglucanase studied with McIlvaine buffer

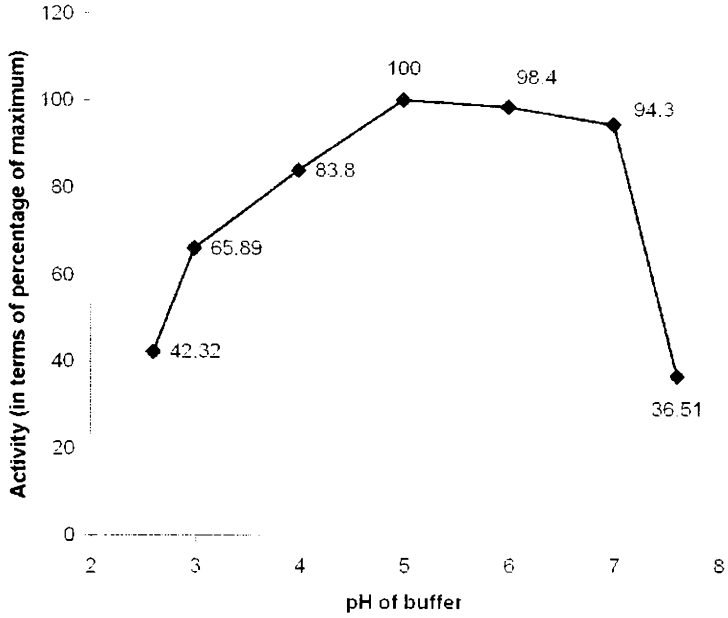


Table 5.5 shows the variation of endoglucanase activity with pH using McIlvaine and glycine–NaOH buffers. Activities in the alkaline pH range had no significance in the determination of optimum pH. The endoglucanase activity is very low in the alkaline pH (using Glycine-NaOH) when compared to that obtained in acid pH (using McIlvaine buffer). Endoglucanases show wide variations in pH optima from species to species. It mainly depends on the source from which the enzyme is isolated.

Most endoglucanases were found to exhibit maximum activity in the pH range 4-6 (Xu et al., 2000; Saha, 2003; Okada, 1975; Marques et al., 2003; Bryne, 1975; Sugimura et al., 2003; Lin & Stutzenberger, 1995; Ng & Zeikus, 1981). Endoglucanases of alkalophilic *Bacillus* sp. N6-27 exhibited maximum activity at pH 8.5 (Tian & Wang, 1998) and that of the cellulase of *Bacillus* sp. KSM-S237 at 8.6-9.0 (Hakamada et al., 1997), are some exceptions.

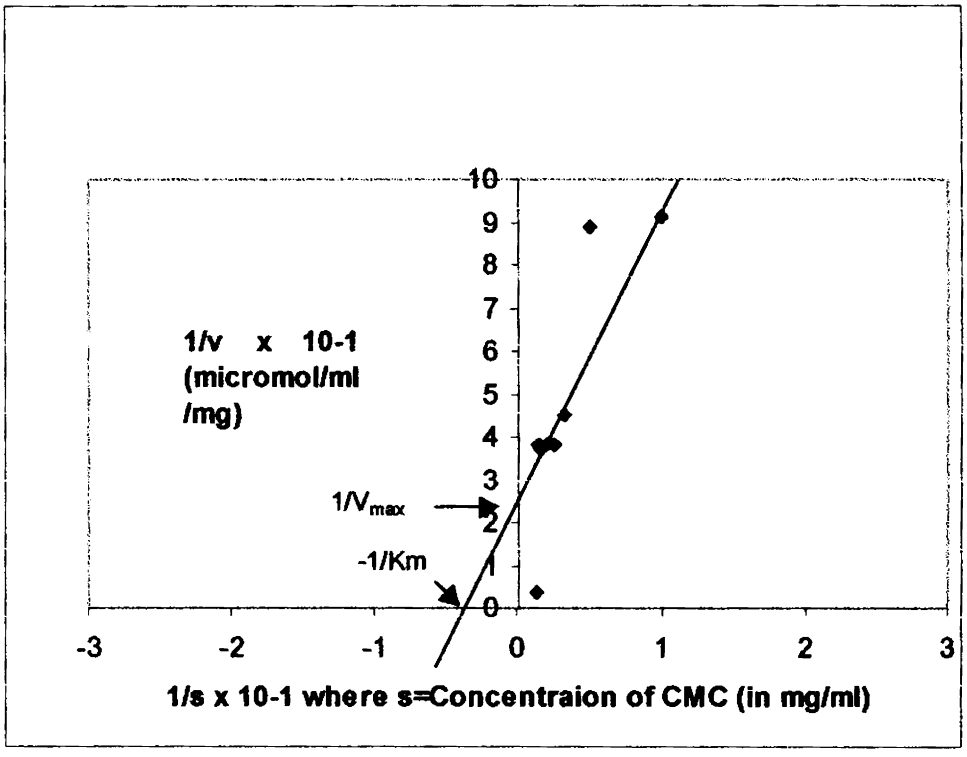
5.8 Effect of substrate concentration on the rate of cellulolysis by the purified green mussel endoglucanase

The endoglucanase enzyme of green mussel actively cleaved soluble cellulose, CMC to glucose. A maximum reaction velocity of 4 μmol/ min/ mg protein was obtained from the Line Weaverburk plot (Figure 5.10). Michaelis – Menten constant K_m was calculated using the equation given in 4.5.2 and a value of 0.26 mg/ml (CMC) was obtained. The K_m value was high for certain endoglucanases i.e., 3.8 mg/ml for *Coptotermes formosanus* endoglucanase (Nakashima & Azuma, 2000) and 3.9 mg/ml for *Thermoascus aurantiacus* endoglucanase (Tong et al., 1980). For certain other bacterial and fungal cellulases the K_m value

Table 5.5 Comparison of the endoglucanase activity obtained at different pH studied with McIlvaine and glycine-NaOH buffers

McIlvaine buffer		Glycine –NaOH buffer	
pH of buffer	Endoglucanase Activity (U)	pH of buffer	Endoglucanase Activity (U)
2.6	0.106	8.6	0.01
3.0	0.165	9.0	0.0
4.0	0.200	10.0	0.0
5.0	0.250		
6.0	0.246		
7.0	0.235		
7.6	0.090		

Figure 5.10 Line Weaver burk plot to show the effect of carboxymethycellulase on the activity of *Perna viridis* Endoglucanase



ranged between 0.3 mg/ml and 0.8 mg/ml (Woolley et al., 2001; Singh & Kumar, 1998). The low K_m value obtained for green mussel endoglucanase (0.26mg/ml) indicate the higher efficiency of the enzyme as K_m is the substrate concentration at half the maximal velocity. The K_m and V_{max} of *Chalara* was 8.3g/L and 1.1μM/min respectively (Lucas et al., 2001).

The kinetic constant V_{max} /K_m for certain endoglucanases studied by different authors are given in Table 5.6. In the present study a V_{max}/K_m of 15.4 is obtained indicating CMC as an effective substrate for green mussel endoglucanase. Higher the kinetic constant, greater is the preference of that enzyme towards the substrate under study. Hence from Table 5.6, it can be concluded that green mussel endoglucanase has more affinity for CMC than *Neisseiria sicca* and the two *Bacillus* endoglucanases studied. However this is less when compared to the endoglucanase of *Thermomonospora curvata*, reported by Lin and Stutzenberger, (1995).

5.9 Isoelectric point of green mussel endoglucanase

The isoelectric point was determined according to the procedure given in 4.5.5. The turbidity of purified green mussel endoglucanase in different pH solutions was visually compared after adding 96% ethanol. The turbidity was then measured using manual photometer SQ118. Table 5.7 shows turbidometric readings corresponding to various pH values. The green mussel endoglucanase was found to be isoelectric at pH 8.8. The endoglucanases studied by different authors show wide variations in the isoelectric point. Isoelectric points of some

Table 5.6 Comparison of Kinetic constant V_{max}/ K_m of green mussel endoglucanase with certain other endoglucanases

Source of Endoglucanase	V_{max}/K_m
Neisseria sicca SB (Moriyoshi et al., 2002)	5.61
Bacillus CH 43 (Mawadza et al., 2000)	0.6
Bacillus HR68 (Mawadza et al., 2000)	1
Thermomonospora curvata (Lin & Stutzenberger, 1995)	113.6
Green mussel (*Perna viridis*)	15.38

Table 5.7: Turbidity values in the determination of isoelectric point of the green mussel Endoglucanase

pH	Turbidity (NTU)
5.0	62
6.0	62
6.6	63
6.8	64
7.0	70
7.2	75
7.4	75
8.0	80
8.6	83
8.8	84
9.0	80

endoglucanases are shown in Table 5.8. The isoelectric point of green mussel endoglucanase is very near to that obtained for the endo-beta-glucanase of psychrotrophic yeast *Rhodotorula glutinis* (8.57) (Oikawa, 1998).

5.10 Effect of ions on the activity of green mussel endoglucanase

The effect of various ions on the 48KDa green mussel endoglucanase studied are summarised in Table 5.9. Certain ions activated (Mn^{2+}, Cd^{2+}, Co^{2+}, I^- and Cl^-) while some others inhibited (Ag^+, Hg^{2+}, Fe^{3+}, Fe^{2+}, Zn^{2+}, Cu^{2+}, Ba^{2+}, Ca^{2+}, Sn^{2+}, Pb^{2+}, Mg^{2+} and Br^-) the activity of green mussel endoglucanase. Of the ions studied, Ag^+, Hg^{2+} and Fe^{3+} exerted maximum inhibition. Hg^{2+} also inhibits various endo-(1, 4) β glucanases from other microorganisms such as *Bacillus* sp. (Mawadza et al., 2000; Singh & Kumar, 1998; Tian & Wang, 1998), *Neisseria sicca SB* (Moriyoshi et al., 2002), *Rhodotorula glutinis* KUJ 2731 (Oikawa, 1998), *Thermomonospora curvata* (Lin & Stutzenberger, 1995) and *Volvariella volvacea* (Ding et al., 2002). Even at low concentrations of 1mM, mercury was found to inhibit endoglucanase activity (Okada, 1975; Creuzet & Frixon, 1983).

The other metal ions showing inhibitory effect on the purified green mussel endoglucanase are $Fe^{2+} > Zn^{2+} > Cu^{2+} = Ca^{2+} > Sn^{2+} > Pb^{2+} > Mg^{2+}$. The inhibitory effect of Zn^{2+}, Fe^{2+}, Mg^{2+}, Ag^+, Hg^{2+} and Pb^{2+} on green mussel endoglucanase shows similarity with that of certain other endoglucanases. Ag^+, Hg^{2+}, Fe^{3+} and Pb^{2+} (250μmol/l) inhibited 70% of the activity of *Thermomonospora curvata* endoglucanase (Lin & Stutzenberger, 1995). However, Zn^{2+} at the same concentration enhanced the enzyme activity by about

Table 5.8: Isoelectric point of certain Endoglucanases

Endoglucanase Source	Isoelectric Point
Bacillus sp. KSM-S 237 Hakamada et al., 1997	3.8
Bacillus N6-27 Tian and Wang, 1998	4.2
Clostridium thermocellum Ng and Zeikus, 1981	6.72
Trichoderma viridae Voragen et al., 1988	6.9
Trichoderma reesei Bhikhabhai et al., 1984	5.5
Trichoderma koningii Wood and McCrae, 1978	4.32
Aspergillus aculeatus Murao et al., 1988	3.4
Aspergillus niger	3.67
Humicola grisea Okada, 1998	6.5
Rhodotorula glutinis Oikawa, 1998	8.57
Green mussel (*Perna viridis*)	8.8

Table 5.9: Effect of ions on green mussel Endoglucanase

Ions	% Activation	% Inhibition
Cl^-	26	-
Br^-	-	21.7
I^-	13.3	-
F^-	-	-
Cu^{2+}	-	47.5
Zn^{2+}	-	56.4
Sn^{2+}	-	30.6
Mn^{2+}	122	-
Mg^{2+}	-	4
Co^{2+}	144	-
Ba^{2+}	-	17.3
Ca^{2+}	-	47.5
Pb^{2+}	-	23.7
Cd^{2+}	64	-
Hg^{2+}	-	Completely inhibited
Fe^{2+}	-	69.9
Fe^{3+}	-	Completely inhibited
Ag^+		Completely inhibited

10%. Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+} and Mg^{2+} showed no significant effect on the activity of *Thermomonospora curvata* endoglucanase. The endoglucanase activity of *Chalara paradoxa* was inhibited by Zn^{2+}, Fe^{2+}, and Mg^{2+} at 10mM concentrations (Lucas et al., 2001). In a study on Molluscan (Asiatic clams and snails) cellulolytic activity response to Zn^{2+} exposure in laboratory, Farris et al., (1994) has reported that exposure of 0.05mg Zn/l or greater significantly reduced endoglucanase activity in both the molluscs. Mg^{2+}, Ca^{2+}, Cu^{2+} and Zn^{2+} were found to inhibit the activity of *Volvariella volvaceae* endoglucanase by varying degrees (Ding et al., 2002).

Heavy metals such as Hg^{2+}, Ag^{+}, Pb^{2+} are non competitive inhibitors. They form mercaptides with –SH groups of enzymes (Jain, 1979).

$$Enz - SH + Ag^{+} \rightleftharpoons Enz - S - Ag + H^{+}$$

Thus in this study, the metals bind with the sulfur containing aminoacids namely, Met and Cys present in endoglucanase there by inhibiting endoglucanase activity.

Mn^{2+} and Co^{2+} ions were found to stimulate the activity of green mussel endoglucanase. Mn^{2+} at 10mM concentration has been found to stimulate endoglucanase activity of hyphomycete *Chalara* (Lucas et al., 2001). The enzyme activity of *Bacillus* endoglucanase was increased by about 38% by Co^{2+} at 1mM concentrations (Mawadza et al., 2000). Co^{2+} decreased the

endoglucanase activities of *Volvariella volvacea* (Ding et al., 2002) and *R. oryzae* (Murashima et al., 2002) by 2% and 67% respectively.

The endoglucanase enzyme of *Mucor circinelloides* (Saha, 2003) enhanced its activity by 27 $\pm$ 5% in presence of Mg^{2+} (5mM) and 44 $\pm$ 14 % in presence of Co^{2+} (0.5mM). The effect of Mg^{2+} ion on this endoglucanase is in contrast to that in the current study for green mussel endoglucanase (4% inhibition) and *V.volvacea* (13% inhibition) (Ding et al., 2002). The inhibitory nature of Mg^{2+} on green mussel endoglucanase agrees with the results obtained for endoglucanase of *Chalara* (Lucas et al., 2001). Cu^{2+} at 10mM concentration extremely inhibited activity of *Rhizopus oryzae* endoglucanase (Murashima et al., 2002). The inhibitory effect of Cu^{2+} on green mussel endoglucanase was 47% while that for *Volvariella volvacea* (Ding et al., 2002) reported was only 14%.

From the data obtained, it could be concluded that the effect of most of the ions on endoglucanase activity of green mussel is similar to that of *Chalara paradoxa* (Lucas et al., 2001) and *Volvariella volvaceae* (Ding et al., 2002).

5.11 Immobilization of green mussel endoglucanase

Immobilized enzymes have received considerable attention because of their advantages over their unimmobilized counterparts as they improve storage and operational, thermal and conformational stabilities and they are easily recovered for reuse (Nascimento et al., 1997; Gawande & Kamat, 1998). Immobilized biocatalysts are freely used in the production of medicines, chemicals, food and

beverages and wastewater treatments (Abdel-Fattah et al., 1997). Among the various means of enzyme immobilization (Gawande & Kamat, 1998; Abdel-Fattah et al., 1997), immobilization by entrapment has recently been used extensively for stability and durability of biocatalysts (Abdel-Naby et al., 1999; Pattanapipitpaisal et al., 2001).

The green mussel endoglucanase was immobilized in polyacrylamide gel and chitosan as described in 4.5.9 and its activity studied. The endoglucanase on polyacrylamide gel retained 66.6% of activity and on chitosan 76%. The endoglucanase of *Arachniotus citrinus* entrapped in polyacryamide was conformationally more stable (Saleem et al., 2004). The immobilized green mussel endoglucanase in polyacrylamide gel and chitosan could be stored at 0°C for 30 days without any significant loss in activity. Endoglucanase of *Trichoderma viridae* has been immobilized on substrate cellulose and used in production of glucose from cellulose. The enzyme, initially soluble when added to the reactor, formed a tight complex with the insoluble cellulose, thus getting immobilized. The carrier was consumed by the enzyme and gets converted to soluble glucose. The enzyme was then reimmobilized by the addition of fresh cellulose (Colowick & Kaplan, 1976)

Thus the above studies reveal that green mussel is a good source of endoglucanase.

In an attempt to study the ability of endoglucanase to degrade paper (photocopier waste, printed waste paper and ordinary paper), it was found that

Perna viridis endoglucanase was highly effective in removing photocopier and printer ink from paper. Endoglucanase mixed with endoxylanase of T. viridae CCMI84 and *Aspergillus terreus* CCMI8 has been used for deinking mixed office waste paper (Marques et al., 2003).

Hydrolytic enzymes, mainly cellulase can selectively hydrolyze cellulose fibre to release ink from the fibre surface and reduce ink particle size. The conventional method of deinking being a difficult one, cellulases can be used in the process. The purified green mussel endoglucanase actively catalyzed the cellulolysis of paper releasing toner and laser printed inks.

The cellulase enzyme system contains endoglucanase (randomly attack amorphous regions of cellulose), cellobiohydrolase (exoenzymes which hydrolyze crystalline cellulose releasing cellobiose) and β-glucosidase (convert cellooligosaccharides and cellobiose to glucose). The synergistic effect of all the three enzymes help in the recycling of paper (Dienes et al., 2002).

Hence the green mussel endoglucanase, being capable of hydrolyzing endo β(1, 4) linkages of cellulose, if supplemented with cellobiohydrolase and β-glucosidase would be effective in hydrolyzing cellulose completely to glucose.

SUMMARY

Green mussel produces more endoglucanase enzyme when compared with the brown mussel. Significant amount of the enzyme is present in the hepatopancreas (56.2% more) in comparison with the whole meat.

A simple method to purify the endoglucanase from hepatopancreas of green mussel has been worked out. Pure endoglucanase was prepared at a yield of 1.7% with 100.2 fold increase in specific activity. The method is described below.

1) Extraction with 0.1M sodium acetate buffer: The digestive gland is homogenized in ice cold 0.1M sodium acetate buffer.
2) Ammonium sulphate fractionation: The extract which contained the protein was fractionally precipitated with ammonium sulphate at 60% saturation. The precipitate containing the endoglucanase was dissolved in 0.1M sodium acetate buffer.
3) Desalting: The dissolved protein was desalted using Sephadex G25.
4) Sephadex G100 chromatography with 50mM imidazole and 50mM EDTA buffer: The endoglucanase obtained after Sephadex G25 column chromatography was subjected to Sephadex G100 chromatography using streamlined elution with 50mM imidazole and 50mM EDTA buffers. The fraction gave only one activity peak corresponding to the endoglucanase protein.

The homogeneity of the purified green mussel endoglucanase was confirmed with SDS PAGE and Sephadex G100 chromatography where in a single band and a single peak respectively was obtained. The green mussel endoglucanase gave a molecular weight of 48KDa on SDS PAGE when compared with both high and low molecular weight markers.

The endoglucanase enzyme exhibited absorption maxima at 280nm indicating the protein nature of the enzyme. Aminoacid composition of the purified green mussel endoglucanase showed glycine in highest amount (12.5%) followed by proline (11.1%), aspartic acid (9.95) and alanine (9%). The ratio of hydrophilic to hydrophobic aminoacids was found to be 1.39, supporting the requirement of water molecules in the catalytic reaction.

The enzyme actively cleaved carboxymethylcellulose to glucose with a K_m and V_{max} of 0.26 mg/ml and 4 μmol/min/mg. The green mussel endoglucanase exhibited maximum activity at 40°C and pH 5. The enzyme was active over a wide range of temperature 30-50°C and pH 4-7. The green mussel endoglucanase was isoelectric at pH 8.8.

Ag^{+}, Hg^{2+} and Fe^{3+} completely inhibited endoglucanase activity probably by non-competitively inhibiting sulphur containing aminoacids. Other inhibitors include $Fe^{2+} > Zn^{2+} > Cu^{2+} = Ca^{2+} > Sn^{2+} > Pb^{2+} > Mg^{2+}$. The inhibitory effect of Mg^{2+} on green mussel endoglucanase is very low. Mn^{2+} and Co^{2+} activated endoglucanase activity. Anions I^{-} and Cl^{-} activated while Br^{-} inhibited endoglucanase activity.

Thus the use of Mn^{2+} and Co^{2+} to enhance endoglucanase activity especially in the industrial application can increase the yield considerably. The enzyme was immobilized on polyacrylamide gel and chitosan and is capable of retaining more that 60% of the original activity. The immobilized endoglucanase was stable and could be stored for 30 days without significant loss in activity.

CONCLUSION

In the attempt to find out catalytic potency and properties of the endoglucanase of green mussel, it could be highlighted that the enzyme is efficient in degrading carboxymethylcellulose to reducing sugars. The immobilized enzyme will find applications in the food industry, paper and pulp industry, wood preservation, alcohol and pharmaceutical industry.

The purification method employed i.e. Sephadex G100 chromatography employing affinity and exclusion principles simplify the purification procedure.

Addition of Mg^{2+} and Co^{2+} at 10mM concentrations enhances endoglucanase activity of green mussel.

The immobilized endoglucanase can be used for deinking mixed office waste paper. The endoglucanase if supplemented with exoglucanase and β-glucosidase under appropriate conditions would help in the recycling of paper.

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Publications:

1. "On Occurrence of certain Biotoxins along the Kerala Coast" - Sona.A, Ashok Kumar, K., Mukundan, M.K., Jugunu, R., Kripa, V., and Gopinathan, C.P. (2003) *In Seafood Safety* published by *Society of Fisheries Technologists (India) and Central Institute of Fisheries Technology, Cochin*, pp. 314 – 320.
2. "Marine Biotoxins – An Overview" - Ashok Kumar, K., Sona. A., and Mukundan. M.K. (2003). *In Seafood Safety* published by *Society of Fisheries Technologists (India) and Central Institute of Fisheries Technology, Cochin*, pp. 302 – 313.